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## Description

### BACKGROUND OF THE INVENTION

#### 5 Field of the Invention

This invention relates to a method of producing glycosylated single-chain pro-urokinase (hereinafter referred to as pro-UK). More particularly, it relates to a method of producing glycosylated pro-UK which comprises recovering mRNA from an established human kidney-derived cell line, preparing cDNA based on  
 10 said mRNA, inserting the cDNA into a vector, introducing the resulting plasmid into an animal cell to thereby produce a transformant, and recovering glycosylated pro-UK from said animal cell.

#### Description of the Prior Art

15 Urokinase is a plasminogen activator obtainable from human urine and can produce excellent therapeutic effects in the treatment of various forms of thrombosis or embolic diseases as well as in the combined use with anticancer agents. Currently, urokinase is produced mainly from human urine by some or other purification method (cf. Williams, J. R. B.: Br. J. Exp. Pathol., 32, 530).

As a result of cell culture technology development in search of a substitute method, the method  
 20 comprising isolating human kidney cells, subculturing the cells and recovering urokinase from the culture is becoming a second important method. However, recent improvements in the recombinant DNA technology have made it possible to produce desired proteins on a commercial scale by using microorganisms such as *Escherichia coli*, *Saccharomyces cerevisiae* and *Bacillus subtilis* or by using cultured mammalian cells [cf. Goeddel, D. V., Itakura, K., et al.: Proc. Natl. Acad. Sci. U.S.A., 76, 106 (1979) and Nagata, S., Taira, S. H.  
 25 and Wissmann, C.: Nature, 284, 1316 (1980)].

### SUMMARY OF THE INVENTION

Under these circumstances, the present inventors studied in an attempt to develop a method of  
 30 producing urokinase using recombinant DNA techniques. As a result, they obtained mRNA from an established human kidney-derived cell line, prepared the cDNA sequence shown in the following sequence (I) by using said mRNA as the template, cultivated animal cells transformed with a plasmid with said cDNA sequence inserted therein and obtained glycosylated pro-UK, and have now completed the present invention.

35 Thus, the present invention relates to a method of producing glycosylated singly-chain pro-urokinase which comprises recovering mRNA from an established human kidney-derived cell line, preparing cDNA based on said mRNA, inserting the cDNA into a vector, introducing the resulting plasmid into an animal cell to thereby produce a transformant, and recovering glycosylated pro-UK from said animal cell.

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## Sequence (I): the base sequence of the structural gene for pro-UK

```

1      10      20
Ser-Asn-Glu-Leu-His-Gln-Val-Pro-Ser-Asn-Cys-Asp-Cys-Leu-Asn-Gly-Gly-Thr-Cys-Val-
AGC AAT GAA CTT CAT CAA GTT CCA TCG AAC TGT GAC TGT CTA AAT GGA GGA ACA TGT GTG

21      30      40
Ser-Asn-Lys-Tyr-Phe-Ser-Asn-Ile-His-Trp-Cys-Asn-Cys-Pro-Lys-Lys-Phe-Gly-Gly-Gln-
TCC AAC AAG TAC TTC TCC AAC ATT CAC TGG TGC AAC TGC CCA AAG AAA TTC GGA GGG CAG

41      50      60
His-Cys-Glu-Ile-Asp-Lys-Ser-Lys-Thr-Cys-Tyr-Glu-Gly-Asn-Gly-His-Phe-Tyr-Arg-Gly-
CAC TGT GAA ATA GAT AAG TCA AAA ACC TGC TAT GAG GGG AAT GGT CAC TTT TAC CGA GGA

61      70      80
Lys-Ala-Ser-Thr-Asp-Thr-Met-Gly-Arg-Pro-Cys-Leu-Pro-Trp-Asn-Ser-Ala-Thr-Val-Leu-
AAG GCC AGC ACT GAC ACC ATG GGC CGG CCC TGC CTG CCC TGG AAC TCT GCC ACT GTC CTT

81      90      100
Gln-Gln-Thr-Tyr-His-Ala-His-Arg-Ser-Asp-Ala-Leu-Gln-Leu-Gly-Leu-Gly-Lys-His-Asn-
CAG CAA ACG TAC CAT GCC CAC AGA TCT GAT GCT CTT CAG CTG GGC CTG GGG AAA CAT AAT

101     110     120
Tyr-Cys-Arg-Asn-Pro-Asp-Asn-Arg-Arg-Arg-Pro-Trp-Cys-Tyr-Val-Gln-Val-Gly-Leu-Lys-
TAC TGC AGG AAC CCA GAC AAC CCG AGG CGA CCC TGG TGC TAT GTG CAG GTG GGC CTA AAG

121     130     140
Pro-Leu-Val-Gln-Glu-Cys-Met-Val-His-Asp-Cys-Ala-Asp-Gly-Lys-Lys-Pro-Ser-Ser-Pro-
CCG CTT GTC CAA GAG TGC ATG GTG CAT GAC TGC GCA GAT GGA AAA AAG CCC TCC TCT CCT

141     150     160
Pro-Glu-Glu-Leu-Lys-Phe-Gln-Cys-Gly-Gln-Lys-Thr-Leu-Arg-Pro-Arg-Phe-Lys-Ile-Ile-
CCA GAA GAA TTA AAA TTT CAG TGT GGC CAA AAG ACT CTG AGG CCC CGC TTT AAG ATT ATT

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(continued on the following page)

161 170 180  
 Gly-Gly-Glu-Phe-Thr-Thr-Ile-Glu-Asn-Gln-Pro-Trp-Phe-Ala-Ala-Ile-Tyr-Arg-Arg-His-  
 GGG GGA GAA TTC ACC ACC ATC GAG AAC CAG CCC TGG TTT GCG GCC ATC TAC AGG AGG CAC  
 181 190 200  
 Arg-Gly-Gly-Ser-Val-Thr-Tyr-Val-Cys-Gly-Gly-Ser-Leu-Ile-Ser-Pro-Cys-Trp-Val-Ile-  
 CGG GGG GGC TCT GTC ACC TAC GTG TGT GGA GGC AGC CTC ATC AGC CCT TGC TGG GTG ATC  
 201 210 220  
 Ser-Ala-Thr-His-Cys-Phe-Ile-Asp-Tyr-Pro-Lys-Lys-Glu-Asp-Tyr-Ile-Val-Tyr-Leu-Gly-  
 AGC GCC ACA CAC TGC TTC ATT GAT TAC CCA AAG AAG GAG GAG TAC ATC GTC TAC CTG GGT  
 221 230 240  
 Arg-Ser-Arg-Leu-Asn-Ser-Asn-Thr-Gln-Gly-Glu-Met-Lys-Phe-Glu-Val-Glu-Asn-Leu-Ile-  
 CGC TCA AGG CTT AAC TCC AAC ACG CAA GGG GAG ATG AAG TTT GAG GTG GAA AAC CTC ATC  
 241 250 260  
 Leu-His-Lys-Asp-Tyr-Ser-Ala-Asp-Thr-Leu-Ala-His-His-Asn-Asp-Ile-Ala-Leu-Leu-Lys-  
 CTA CAC AAG GAC TAC AGC GCT GAC ACG CTT GCT CAC CAC AAC GAC ATT GCC TTG CTG AAG  
 261 270 280  
 Ile-Arg-Ser-Lys-Glu-Gly-Arg-Cys-Ala-Gln-Pro-Ser-Arg-Thr-Ile-Gln-Thr-Ile-Cys-Leu-  
 ATC CGT TCC AAG GAG GGC AGG TGT GCG CAG CCA TCC CGG ACT ATA CAG ACC ATC TGC CTG  
 281 290 300  
 Pro-Ser-Met-Tyr-Asn-Asp-Pro-Gln-Phe-Gly-Thr-Ser-Cys-Glu-Ile-Thr-Gly-Phe-Gly-Lys-  
 CCC TCG ATG TAT AAC GAT CCC CAG TTT GGC ACA AGC TGT GAG ATC ACT GGC TTT GGA AAA  
 301 310 320  
 Glu-Asn-Ser-Thr-Asp-Tyr-Leu-Tyr-Pro-Glu-Gln-Leu-Lys-Met-Thr-Val-Val-Lys-Leu-Ile-  
 GAG AAT TCT ACC GAC TAT CTC TAT CCG GAG CAG CTG AAG ATG ACT GGT GTG AAG CTG ATT  
 321 330 340  
 Ser-His-Arg-Glu-Cys-Gln-Gln-Pro-His-Tyr-Tyr-Gly-Ser-Glu-Val-Thr-Thr-Lys-Met-Leu-  
 TCC CAC CGG GAG TGT CAG CAG CCC CAC TAC TAC GGC TCT GAA GTC ACC ACC AAA ATG CTG

(continued on the following page)

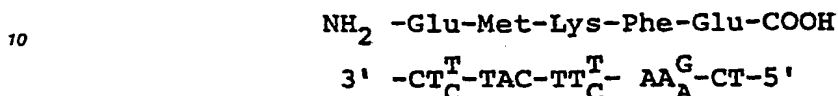
341 Cys-Ala-Ala-Asp-Pro-Gln-Trp-Lys-Thr-Asp-Ser-Cys-Gln-Gly-Asp-Ser-Gly-Gly-Pro-Leu- 360  
 TGT GCT GCT GAC CCA CAG TGG AAA ACA GAT TCC TGC CAG GGA GAC TCA GGG GGA CCC CTC  
 350  
 361 Val-Cys-Ser-Leu-Gln-Gly-Arg-Met-Thr-Leu-Thr-Gly-Ile-Val-Ser-Trp-Gly-Arg-Gly-Cys- 380  
 GTC TGT TCC CTC CAA GGC CGC ATG ACT TTG ACT GGA ATT GTG ACC TGG GGC CGT GGA TGT  
 370  
 381 Ala-Leu-Lys-Asp-Lys-Pro-Gly-Val-Tyr-Thr-Arg-Val-Ser-His-Phe-Leu-Pro-Trp-Ile-Arg- 400  
 GCC CTG AAG GAC AAG CCA GGC GTC TAC ACG AGA GTC TCA CAC TTC TTA CCC TGG ATC CGC  
 390  
 401 Ser-His-Thr-Lys-Glu-Glu-Asn-Gly-Leu-Ala-Leu 410  
 AGT CAC ACC AAG GAA GAG AAT GGC CTG GCC CTC

The pro-UK according to the invention was produced in the following manner:

Using a mRNA fraction (185 to 20S) containing urokinase mRNA as isolated and purified from an established, human kidney-derived cell line, cDNA was synthesized. Starting with 20 µg of mRNA and using reverse transcriptase and DNA polymerase I, there was obtained 3.3 µg of double-stranded cDNA (ds cDNA). After removal of the single-strand d portion by treatment with S1 nuclease, the double stranded cDNA was subjected to sucrose density gradient centrifugation to thereby collect a large molecular ds cDNA fraction (750 ng). A 1/5 portion (150 ng) of this double-stranded cDNA was subjected to treatment for

adding a poly(C) tail. A poly(G) tail was added to the *Escherichia coli* plasmid pBR322 prepared separately. Both the addition products were joined together by annealing. *Escherichia coli* was transformed with the pBR322 plasmid containing the ds cDNA inserted therein, giving about 70,000 transformants, over 90% of which showed ampicillin resistance indicative of the insertion of the cDNA at the PstI site of pBR322.

- 5 Of these transformants, 10,000 colonies were subjected to screening for selecting urokinase cDNA. Based on the amino acid sequence of urokinase, a mixture of eight 14-mer oligodeoxyribonucleotides, namely



- was synthetically prepared and used as a hybridization probe in screening for urokinase cDNA by colony hybridization. As a result, four positive colonies expected to contain urokinase cDNA were obtained. Plasmid DNAs (pUK1 through 4) were prepared from these four colonies and measured for the size of cDNA. pUK1, pUK2, pUK3 and pUK4 were found to contain 1900 bp, 170 bp, 1150 bp and 1300 bp cDNAs, respectively. Detailed restriction enzyme cleavage maps were prepared for pUK1, pUK2 and pUK3, whereby these cDNAs gave restriction maps overlapping one another (cf. Fig. 1). The partial DNA base sequence each of pUK1 and pUK4 as determined by the Maxam-Gilbert method was in agreement with the DNA base sequence deduced from the known amino acid sequence of urokinase. pUK1 had about 670 bp-long 3'-noncoding region-containing cDNA whereas pUK4 had a signal peptide and 5'-noncoding region-containing cDNA. These pUK1 and pUK4 were joined together taking advantage of the BglII site to thereby construct a plasmid containing the entire translation region for urokinase. Using this plasmid, the DNA base sequence of the entire urokinase translation region and its neighborhood was determined and, based on said sequence, the amino acid sequence of the signal peptide and translation region was further determined [hereinafter referred to as sequence (II)].
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Sequence (II): the base sequence of the signal sequence and noncoding region of  
the pro-UK structural gene and the corresponding amino acid sequence

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5' TCCACCTGTCCCCGACGGCGGCTCGCGCCCTCTCTGCGCAGCCACCGAGCCGCGCTGAGGCCCGACCTCGCCACC
-20      Met Arg Ala Leu Leu Ala Arg Leu Leu Leu Cys Val Leu Val Val Ser Asp Ser Lys Gly
      ATG AGA GCC CTG CTG GCG CGC CTG CTT CTC TGC GTC GTG AGC GAC TCC AAA GGC
-10      Ser-Asn-Glu-Leu-His-Gln-Val-Pro-Ser-Asn-Cys-Asp-Cys-Leu-Asn-Gly-Gly-Thr-Cys-Val-
      AGC AAT GAA CTT CAT CAA GTT CCA TCG AAC TGT GAC TGT CTA AAT GGA GGA ACA TGT GTG
1      10      20      30      40      50      60      70      80      90      100
Ser-Asn-Lys-Tyr-Phe-Ser-Asn-Ile-His-Trip-Cys-Asn-Cys-Pro-Lys-Lys-Phe-Gly-Gly-Gln-
21      TCC AAC AAG TAC TTC TCC AAC ATT CAC TGG TGC AAC TGC CCA AAG AAA TTC GGA GGG CAG
41      His-Cys-Glu-Ile-Asp-Lys-Ser-Lys-Thr-Cys-Tyr-Glu-Gly-Asn-Gly-His-Phe-Tyr-Arg-Gly-
      CAC TGT GAA ATA GAT AAG TCA AAA ACC TGC TAT GAG GGG AAT GGT CAC TTT TAC CGA GGA
61      Lys-Ala-Ser-Thr-Asp-Thr-Met-Gly-Arg-Pro-Cys-Leu-Pro-Trip-Asn-Ser-Ala-Thr-Val-Leu-
      AAG GCC AGC ACT GAC ACC ATG GGC CGG CCC TGC CTG CCC TGG AAC TCT GCC ACT GTC CTT
81      Gln-Gln-Thr-Tyr-His-Ala-His-Arg-Ser-Asp-Ala-Leu-Gln-Leu-Gly-Leu-Gly-Lys-His-Asn-
      CAG CAA ACG TAC CAT GCC CAC AGA TCT GAT GCT CTT CAG CTG GGC CTG GGG AAA CAT AAT
101     110     120
Tyr-Cys-Arg-Asn-Pro-Asp-Asn-Arg-Arg-Arg-Pro-Trip-Cys-Tyr-Val-Gln-Val-Gly-Leu-Lys-
TAC TGC AGG AAC CCA GAC AAC CGG AGG CGA CCC TGG TGC TAT GTG CAG GTG GGC CTA AAG

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(continued on the following page)

121 130 140  
 Pro-Leu-Val-Gln-Glu-Cys-Met-Val-His-Asp-Cys-Ala-Asp-Gly-Lys-Pro-Ser-Ser-Pro-  
 CCG CTT GTC CAA GAG TGC ATG CAT GAG TGC GCA GAT GGA AAA AAG CCC TCC TCT CCT  
 141 150 160  
 Pro-Glu-Glu-Leu-Lys-Phe-Gln-Cys-Gly-Gln-Lys-Thr-Leu-Arg-Pro-Arg-Phe-Lys-Ile-Ile-  
 CCA GAA GAA TTA AAA TTT CAG TGT GGC CAA AAG ACT CTG AGG CCC CGC TTT AAG ATT ATT  
 161 170 180  
 Gly-Gly-Glu-Phe-Thr-Thr-Ile-Glu-Asn-Gln-Pro-Trp-Phe-Ala-Ile-Tyr-Arg-Arg-His-  
 GGG GGA GAA TTC ACC ACC ATC GAG AAC CAG CCC TGG TTT GCG GCC ATC TAC AGG AGG CAC  
 181 190 200  
 Arg-Gly-Gly-Ser-Val-Thr-Tyr-Val-Cys-Gly-Gly-Ser-Leu-Ile-Ser-Pro-Cys-Trp-Val-Ile-  
 CGG GGG GGC TCT GTC ACC TAC GTG TGT GGA GGC AGC CTC ATC AGC CCT TGC TGG GTG ATC  
 201 210 220  
 Ser-Ala-Thr-His-Cys-Phe-Ile-Asp-Tyr-Pro-Lys-Lys-Glu-Asp-Tyr-Ile-Val-Tyr-Leu-Gly-  
 AGC GCC ACA CAC TGC TTC ATT GAT TAC CCA AAG AAG GAG GAC TAC ATC GTC TAC CTG GGT  
 221 230 240  
 Arg-Ser-Arg-Leu-Asn-Ser-Asn-Thr-Gln-Gly-Glu-Met-Lys-Phe-Glu-Val-Glu-Asn-Leu-Ile-  
 CGC TCA AGG CTT AAC TCC AAC ACG CAA GGG GAG ATG AAG TTT GAG GTG GAA AAC CTC ATC  
 241 250 260  
 Leu-His-Lys-Asp-Tyr-Ser-Ala-Asp-Thr-Leu-Ala-His-His-Asn-Asp-Ile-Ala-Leu-Leu-Lys-  
 CTA CAC AAG GAC TAC AGC GCT GAC ACG CTT GCT CAC CAC AAC GAC ATT GCC TTG CTG AAG  
 261 270 280  
 Ile-Arg-Ser-Lys-Glu-Gly-Arg-Cys-Ala-Gln-Pro-Ser-Arg-Thr-Ile-Gln-Thr-Ile-Cys-Leu-  
 ATC CGT TCC AAG GAG GGC AGG TGT GCG CAG CCA TCC CGG ACT ATA CAG ACC ATC TGC CTG

(continued on the following page)



281 300  
 Pro-Ser-Met-Tyr-Asn-Asp-Pro-Gln-Phe-Gly-Thr-Ser-Cys-Glu-Ile-Thr-Gly-Phe-Gly-Lys-  
 CCC TCG ATG TAT AAC GAT CCC CAG TTT GGC ACA AGC TGT GAG ATC ACT GGC TTT GGA AAA  
 290  
 301 310  
 Glu-Asn-Ser-Thr-Asp-Tyr-Leu-Tyr-Pro-Gln-Gln-Leu-Lys-Met-Thr-Val-Val-Lys-Leu-Ile-  
 GAG AAT TCT ACC GAC TAT CTC TAT CCG GAG CAG CTG AAG ATG ACT GTT GTG AAG CTG ATT  
 320  
 321 330  
 Ser-His-Arg-Glu-Cys-Gln-Gln-Pro-His-Tyr-Tyr-Gly-Ser-Glu-Val-Thr-Thr-Lys-Met-Leu-  
 TCC CAC CGG GAG TGT CAG CAG CCC CAC TAC TAC GGC TCT GAA GTC ACC ACC AAA ATG CTG  
 340  
 341 350  
 Cys-Ala-Ala-Asp-Pro-Gln-Trp-Lys-Thr-Asp-Ser-Cys-Gln-Gly-Asp-Ser-Gly-Gly-Pro-Leu-  
 TGT GCT GCT GAC CCA CAG TGG AAA ACA GAT TCC TGC CAG GGA GAC TCA GGG GGA CCC CTC  
 360  
 361 370  
 Val-Cys-Ser-Leu-Gln-Gly-Arg-Met-Thr-Leu-Thr-Gly-Ile-Val-Ser-Trp-Gly-Arg-Gly-Cys-  
 GTC TGT TCC CTC CAA GGC CGC ATG ACT TTG ACT GGA ATT GTG AGC TGG GGC CGT GGA TGT  
 380  
 381 390  
 Ala-Leu-Lys-Asp-Lys-Pro-Gly-Val-Tyr-Thr-Arg-Val-Ser-His-Phe-Leu-Pro-Trp-Ile-Arg-  
 GCC CTG AAG GAC AAG CCA GGC GTC TAC ACG AGA GTC TCA CAC TTC TTA CCC TGG ATC CGC  
 400  
 401 410  
 Ser-His-Thr-Lys-Glu-Glu-Asn-Gly-Leu-Ala-Leu Stop  
 AGT CAC ACC AAG GAA GAG AAT GGC CTG GCC CTC TGA GGGTCCCCAGGAGGAACGGGCACACCCGC  
 TTTTCTGCTGCTGCTATTTTTCAGTAGAGTCACTCTCCATCAGCTGTAAGAAGAGACTGGGAAGA .....3'

It was thus revealed that urokinase is constituted by a signal peptide composed of 20 amino acids and the  
 subsequent 411 amino acids. Furthermore, the DNA base sequence of urokinase cDNA and other data  
 revealed that a 5'-noncoding region comprising not less than 80 bases and a 3'-noncoding region  
 55 comprising not less than 800 bases occur in urokinase mRNA [cf. Sequence (II)].

The thus-obtained urokinase cDNA was inserted into a vector usable in a heterologous gene expression  
 system in animal cells, followed by introduction into animal cells. Production of glycosylated pro-UK with  
 molecular weight of 54,000 dalton in the transformant cells thus obtained was confirmed.

BRIEF DESCRIPTION OF THE DRAWING

In the accompanying drawings,

- Fig. 1 shows the restriction enzyme map of cDNA;
- 5 Fig. 2 shows the ultracentrifugation sedimentation pattern of cDNA;
- Fig. 3 shows the structure of pro-UK mRNA and the position of the synthetic DNA probe;
- Fig. 4 shows the restriction enzyme map each of pUK1, pUK4 and pUK18, pUK1 having cDNA of about 1,900 bp, pUK4 having cDNA of about 1,300 bp and pUK18 having cDNA of about 1,450 bp, their restriction enzyme maps overlapping with one another;
- 10 Fig. 5 shows the restriction enzyme map of cDNA, wherein the restriction enzyme of cDNA is given in a unified form on the basis of Fig. 7; \*, X and O indicating the position of [<sup>32</sup>P] labelling and the arrow indicating the direction of sequencing; \*, X and O meaning that the sequencing was performed using pUK1, pUK4 and pUK18, respectively;
- Fig. 6 shows the flowchart for the construction of plasmid pUK33 by ligation of pUK1 with pUK4, wherein
- 15 E, H and B stand for EcoRI, HindIII and BamHI, respectively;
- Fig. 7 shows the flowchart for the ligation of pcDV1 with pL1;
- Fig. 8 shows the restriction enzyme map of pSVI-GI;
- Fig. 9 shows the restriction enzyme map of pSV-GI-preUK; and pSV-GI-NEO;
- Fig. 10 shows the structure of preUK cDNA and its neighborhood in pSV-GI-preUK, wherein NC, SS and
- 20 SJ stand for noncoding region, signal sequence and splicing junction, respectively;
- Fig. 11 shows the results in SDS-PAGE for measurement of the molecular weight of urokinase-like protein in the culture supernatant.
- lane 1,6 : Urinary urokinase
- lane 2 : Culture supernatant of human kidney-derived cell line. (54k in molecular weight band was
- 25 detected.)
- lane 3,4,5 : Culture supernatant of CHD-K<sub>1</sub>, C-68 and C-61 cells. (54k, 34k and 20k in molecular weight bands were detected.)

DETAILED DESCRIPTION OF THE INVENTION

- 30 (1) Isolation and purification of mRNA
- (a) Cells used
- For extraction of whole RNA from human kidney-derived established cell line capable of producing
- 35 urokinase, these cells were first allowed to form a monolayer in a multistage plastic incubator and a Roux flask using a growth medium. Thereafter, the medium was changed for a maintenance medium and the cultivation was continued for further 10-15 hours. 2 to 5 x 10<sup>9</sup> cells at that stage were collected by trypsin treatment, followed by whole RNA extraction.
- (b) Recovery of mRNA
- 40 Using the method disclosed in Japanese Tokkyo Kokai Koho 58-148899 (filed by Green Cross Corp.), mRNA having a size of 19-20S with which urokinase activity was noted upon injection into Xenopus laevis oocytes, was isolated and purified.
- (2) Synthesis of single-stranded cDNA
- 45 The synthesis of cDNA was performed by the method of Maruin, P. W. et al.: J. Biol. Chem., 253, 2483-2495 (1978).
- (a) To the reaction mixture specified in Table 1, there was added 20 µg of urokinase mRNA heated at
- 50 65° C for 5 minutes and rapidly cooled on ice. After stirring well in an Eppendorff tube, the mixture was allowed to stand on ice.

Table 1

	Reaction mixture	Amount	Final concentration
5	0.5M Tris-HCl (pH8.3)	20 $\mu$ l	50mM
10	1.4M KCl	10 $\mu$ l	70mM
	0.25M $MgCl_2$	8 $\mu$ l	10mM
15	0.05M dNTP.	Each 2 $\mu$ l	Each 0.5mM
	50 $\mu$ Ci $\alpha$ - $^{32}$ P-dCTP	10 $\mu$ l	
	0.2M DTT	10 $\mu$ l	10mM
20	Oligo (dt) <sub>12-18</sub> (250 $\mu$ g/ml)	20 $\mu$ l	25 $\mu$ g/ml
25	mRNA	10 or 20 $\mu$ g	
	AMV reverse transcriptase	160 units	800 u/ml
	RNase inhibitor	22 units	70 u/ml
30	H <sub>2</sub> O	To make the Total 200 $\mu$ l	

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(b) Single-stranded cDNA was synthesized by reaction at 46° C for 10-12 minutes.

(c) The reaction was terminated by adding 20  $\mu$ l of 0.5 M EDTA to the reaction mixture.

40 (d) After extraction with 200  $\mu$ l of phenol-chloroform, the mixture was centrifuged at 10,000 rpm for 3 minutes. The aqueous layer was separated. The lower phenol layer was reextracted with an equal amount of Sephadex G-100 buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 100 mM NaCl). Both the aqueous layers were combined and 60  $\mu$ l of 80% glycerin was added to the resulting reaction mixture.

(e) The reaction mixture was applied to a Sephadex G-100 column, followed by elution with G-100 buffer. The eluate was fractionated by 5 drops.

45 (f) Each fraction was tested by means of a Cherenkov counter and radioactive peak portions were collected.

(g) Thereto were added 1/10 volume of 3 M sodium acetate buffer, 10  $\mu$ g of tRNA and 2 volumes of ethanol, and the mixture was cooled at -80° C for 20 minutes.

(h) The precipitate was collected by centrifugation (15,000 rpm, 10 minutes) and dried under reduced pressure.

50 (i) The precipitate was dissolved in 300  $\mu$ l of 0.1 N aqueous NaOH, followed by incubation at 70° C for 20 minutes and cooling with water.

(j) The solution was neutralized by adding about 30  $\mu$ l of 1 N HCl. Thereafter, a 2  $\mu$ l portion was sampled and measured for radioactivity.

55 (k) 10  $\mu$ g of tRNA was added, followed by precipitation with ethanol. The precipitate was collected by centrifugation and dried under reduced pressure.

(l) The synthesis of single-stranded cDNA was thus completed. The yield of single-stranded cDNA was determined by recovering single-stranded cDNA-mRNA hybrid by G-100 column chromatography and degradative removal of mRNA by hot alkali treatment. As a result, 3.65  $\mu$ g of urokinase-encoding singl -

strand d cDNA was obtained.

### (3) Synthesis of second strand DNA

For the synthesis of second strand DNA, DNA was first synthesized using DNA polymerase I and then the DNA chain was extended using reverse transcriptase.

(a) 100  $\mu$ l of single-stranded cDNA was suspended in 2 x reaction mixture (Table 2).

Table 2

Reaction mixture	Amount	Concentration
0.5 M HEPES-NaOH (pH6.9)	20 $\mu$ l	100 mM
67 mM $MgCl_2$		13.4 mM
0.7 M KCl		140 mM
0.2 M DTT	5 $\mu$ l	10 mM
5 mM dXTP	20 $\mu$ l	1 mM
H <sub>2</sub> O	To make the total 100 $\mu$ l	

(b) Incubation was conducted at 60°C for 2 minutes, followed by centrifugation (15,000 rpm, 2.5 minutes). The supernatant was collected.

(c) Following addition of 50  $\mu$ l of water to the precipitate, the procedure of (b) was repeated. Both the supernatants were combined.

(d) 50 units of DNA polymerase I Klenow fragment was added and the total amount was made 200  $\mu$ l.

(e) Incubation was performed at 15°C overnight.

(f) The reaction was terminated by adding 20  $\mu$ l of 0.2 M EDTA (pH 8.0), then an equal volume (220  $\mu$ l) of phenol-chloroform solution was added and the mixture was stirred and centrifuged. The aqueous layer was collected. The phenol layer was reextracted with 200  $\mu$ l of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA).

(g) Both the aqueous layers were combined and 60  $\mu$ l of 80% glycerin was added.

(h) The resulting mixture was applied to a Sephadex G-100 column, followed by elution with G-100 buffer. The eluate was fractionated by 5 drops.

(i) Each fraction was tested by means of a Cherenkov counter, and radioactivity peak fractions were collected. 20  $\mu$ l aliquot was mixed with 10 ml of ACS II and the mixture was used for radioactivity measurement. To the remaining portion of the liquid, there were added 5  $\mu$ g of tRNA and 1/10 volume of 3 M sodium acetate buffer, followed by precipitation with ethanol.

(j) The precipitate was collected by centrifugation (15,000 rpm, 10 minutes) and dried under reduced pressure.

There was thus obtained 5.3  $\mu$ g of ds cDNA.

Thereafter, synthetic extension of second strand DNA was conducted using reverse transcriptase.

### (4) Synthetic extension of second strand DNA using reverse transcriptase

(a) The ds cDNA obtained was dissolved in 20  $\mu$ l of water. To the solution was added 30  $\mu$ l of the reaction mixture given in Table 3, followed by stirring.

Table 3

	Reaction mixture	Amount
5	1 M Tris-HCl (pH 8.3)	5 $\mu$ l
10	1M KCl	7 $\mu$ l
	250 mM MgCl <sub>2</sub>	2 $\mu$ l
15	dNTP (20 mM)	2.5 $\mu$ l
	700 mM $\beta$ -mercaptoethanol	2 $\mu$ l
	AMV reverse transcriptase	2 $\mu$ l
20	H <sub>2</sub> O	To make the total 30 $\mu$ l

- 26 (b) This mixture was incubated at 42° C for 60 minutes.  
 (c) The reaction was terminated by adding 5  $\mu$ l of 0.2 M EDTA.  
 (d) Deproteinization was performed by adding an equal volume (55  $\mu$ l) of phenol-chloroform.  
 (e) Thereafter, the same procedures [(g)-(j)] as in the synthesis of ds cDNA using polymerase I were performed, followed by digestion treatment of the single-stranded DNA portion with SI nuclease according to Shenk, T. E. et al. [cf. Proc. Natl. Acad. Sci. U.S.A., 72, 989 (1975)], as mentioned below.

(5) Digestion of single-stranded DNA portion with SI nuclease

- 35 (a) The ds cDNA was suspended in 100  $\mu$ l of buffer for SI nuclease [0.3 M NaCl, 30 mM sodium acetate (pH 4.5), 3 mM ZnCl<sub>2</sub>] and, after complete dissolution of ds cDNA by incubation at 37° C for 30 minutes, 0.5 units of SI nuclease (product of PL Biochemical) was added.  
 (b) After 30 minutes of reaction at 37° C, 2 units of SI nuclease was further added and the reaction was allowed to proceed for further 15 minutes.  
 (c) 20  $\mu$ l of 0.2 M EDTA was added to terminate the reaction, followed by addition of 120  $\mu$ l of phenol-chloroform for deproteinization.  
 40 (d) The phenol layer was reextracted with TE buffer. Both the aqueous layers were combined and subjected to precipitation with ethanol to give ds cDNA.

(6) Sucrose density gradient centrifugation of ds cDNA

- 45 The ds cDNA obtained was then subjected to sucrose density gradient centrifugation (38,000 rpm, 4° C) and large molecular weight fractions were collected. The ultracentrifugation sedimentation pattern of urokinase ds cDNA is shown in Fig. 2 (indicated by ● - ● in the figure), together with the sedimentation pattern of globin ds cDNA as a marker (indicated by ▲ - ▲ in the figure). Urokinase ds cDNA shows a slight shift of the peak fraction to the bottom as compared with globin ds cDNA. Urokinase ds cDNA was also more abundant in large ds cDNA in fractions 11 to 15 and the neighboring fractions. Therefore, fractions 6-15 were collected and used as large ds cDNA. This large ds cDNA weighed 722 ng.

(7) Addition of poly(C) tail to ds cDNA

- 55 The C-tail addition reaction was conducted on the assumption that urokinase ds cDNA is on an average 1,500 bp in size. The C-tail reaction should ideally be conducted such that a poly(C) tail comprising 15 to 20 dCs is added to the 3' OH terminal of ds cDNA. The inventors used 5 units of terminal transferase and

carried out the reaction at 37°C. As a result, about 20 dCs were added as the C-tail.

In the same manner, a poly(G) tail comprising about 15 dGs was added to the PstI site of *Escherichia coli* plasmid pBR322 prepared separately. Equimolar amounts of both products were mixed and annealed under high ion strength conditions by gentle cooling from 70°C to 37°C.

#### (8) Transformation

The *Escherichia coli* RRI was transformed with the annealed DNA, giving about 70,000 tetracycline-resistant strains. The transformants obtained were examined for ampicillin susceptibility by the replica method. More than 90% of the transformants, i.e. about 60,000 colonies, exhibited Ap<sup>r</sup> (Ampicillin susceptibility) and Tc<sup>r</sup> (Tetracycline-resistance), suggesting the insertion of cDNA.

#### (9) Cloning of urokinase gene

For cloning urokinase cDNA, an oligodeoxyribonucleotide mixture was synthesized based on the known amino acid sequence of Urinary Urokinase and transformant screening was conducted using this as a hybridization probe.

##### (a) Synthetic oligodeoxyribonucleotide probe

The 14-base synthetic oligodeoxyribonucleotide mixture used as a probe (hereinafter referred to as probe) was custom-synthesized by Nippon Zeon Co., Ltd. (Tokyo).

The nucleotide sequence of the probe used and the corresponding urokinase nucleotide sequence are shown in Table 4. The probe was drawn from the 73rd to 77th amino acids of human urine urokinase B chain. As is evident in Table 4, the probe is a mixture of 8 oligonucleotides.

**Table 4    Oligonucleotide probe used for  
isolating human urokinase cDNA**

Amino acid sequence	73	74	75	76	77
NH <sub>2</sub> -Glu-Met-Lys-Phe-Glu-COOH					
Codon	5'-GA <sup>G</sup> <sub>A</sub> -AUG-AA <sup>G</sup> <sub>A</sub> -UU <sup>U</sup> <sub>C</sub> -GA <sup>G</sup> <sub>A</sub> -3'				
14-mer mixture	3'-CT <sup>T</sup> <sub>C</sub> -TAC-TT <sup>T</sup> <sub>C</sub> -AA <sup>G</sup> <sub>A</sub> -CT-5'				

Note: In the middle line is shown all possible sequences of mRNA. In the lower line is shown the base sequence of the probe DNA complementary to said mRNA. The probe DNA is lacking in the third base in the codon for the 77th amino acid (Glu).

##### (b) Preparation of probe labelled with <sup>32</sup>P at 5' end

###### 1) <sup>32</sup>P labelling of probe at 5' end

The 5' end of the probe was labelled with [γ-<sup>32</sup>P] ATP (Amersham, Specific activity 5,000 ci/mMol) and T4 polynucleotide kinase. As regards the labelling conditions, the mole ratio of <sup>32</sup>P-ATP to 5'-OH end was 5:1 and the reaction system was as follows:

Reaction mixture:

	Probe DNA	6-80 pmol 5'-OH end
	*1 10 x kinase buffer	2 $\mu$ l
5	T4 polynucleotide kinase	1 $\mu$ l
	*2 [ $\gamma$ - <sup>32</sup> P]ATP	5 x mol of 5'-OH end
10	H <sub>2</sub> O	To make the total 20 $\mu$ l

\*1 10 x kinase buffer:

	0.7 M Tris-HCl (pH 7.6)
15	0.1 M MgCl <sub>2</sub>
	50 mM DTT

20 The reaction was carried out at 37° C for 60-120 minutes. After completion of the reaction, 1  $\mu$ l of 0.5 M EDTA (pH 8.0), 35  $\mu$ l of 1M sodium chloride (in 10 mM Tris-HCl buffer and 1 mM EDTA) and 10.5  $\mu$ l of 1 mg/ml tRNA solution and the final volume was made 35  $\mu$ l, and the mixture was heat-treated at 70° C for 3 minutes and then applied to a NACS-52 minicolumn.

ii) Purification of <sup>32</sup>P-labelled probe

25 The <sup>32</sup>P-labelled probe was purified by applying the reaction mixture prepared in i) on to a NACS-52 minicolumn (BRL).

The efficiency of labelling was almost constant irrespective of the kind of probe. Supposing that the recovery from the column was 80%, the specific activity of the probe DNA was estimated at about 1.0 x 10<sup>9</sup> cpm/ $\mu$ g of DNA.

30 (c) Colony hybridization

Using the probe mentioned above, screening for urokinase cDNA was conducted by colony hybridization.

i) The reagents and buffers used were as follows: Deionized formamide:

35 Formamide (500 ml) and Mixed-bed-resin (30 ml) were mixed and the mixture was stirred at 4° C for 2 hours, suction-filtered and stored at -80° C.

50 x Denhardt's solution:

Stored at -20° C after bacterial filtration.

20 x SSPE:

	3.6 M NaCl
40	0.2 M NaH <sub>2</sub> PO <sub>4</sub> (adjusted to pH 7.4 with 10 N NaOH)
	20 mM EDTA
	20 x SSC

Sonicated salmon sperm DNA

Prehybridization washing solution (hereinafter, PHWS):

45 50 mM Tris-HCl (pH 8.0)

1 M NaCl

1 mM EDTA

0.1% SDS

Prehybridization solution:

50 50% deionized formamide

5 x Denhardt's solution

5 x SSPE

0.1% SDS

100  $\mu$ g/ml Sonicated DNA

55 Washing solution I (hereinafter, WS I):

5 x SSC

1 x Denhardt's solution

0.1% SDS

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graph TD
    A[15 Dry filter] --> B[20 Washing (moistening the filter with PHWS at 42°C for  
1 to 2 hours)]
    B --> C[25 Prehybridization (42°C, 4 to 6 hours)]
    C --> D[30 Washing twice with WS I]
    D --> E[35 Hybridization (37°C, 20 hours, in 32P-labelled probe-  
containing HS)]
    E --> F[40 Washing with WS II (room temperature, 3 minutes, three  
times; then, 37°C, 3 minutes, three  
times)]
  
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Using  $3.0 \times 10^8$  cpm of the  $^{32}\text{P}$ -labelled probe, 10,000 colonies (100 filters) were screened. The total amount of HS was 150 ml and therefore the probe concentration was  $2.0 \times 10^6$  cpm/ml. The hybridization was conducted at  $37^\circ\text{C}$  for 21 hours with occasional shaking. Thereafter, the filter was washed with WS II three times at room temperature and then three times at  $37^\circ\text{C}$ . The dried filter was placed on a filter paper (Whatman 3MM), and autoradiography was performed at  $-70^\circ\text{C}$  for 2 days, followed by development. As a result, 65 colonies seemingly indicative of relatively high rate of hybridization were selected and again fixed on a filter for rescreening.

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the probe DNA concentration being  $5.8 \times 10^5$  cpm/ml/oligonucleotide species.

After 21 hours of hybridization at  $37^\circ\text{C}$ , the filters were washed with WS II. One filter was dried at  $37^\circ\text{C}$  directly after washing, while the other was further washed three times at  $42^\circ\text{C}$  for 3 minutes, followed by exposure of an X-ray film thereto.

5 In this hybridization experiments, four colonies were found to be indicative of hybridization with the probe DNA. After washing at  $42^\circ\text{C}$ , these 4 colonies were still clearly recognized as positive colonies.

For the 4 colonies selected, plasmid DNAs (named pUK1, 2, 3 and 4, respectively) were prepared and examined for the size of cDNA. Among the 4 plasmids pUK1 had the largest cDNA of about 1,900 bp. pUK3 and pUK4 were smaller than pUK1 and were about 1,300 bp and about 1,150 bp in size, respectively. pUK2  
10 was the smallest cDNA about 170 bp in size. Since, in the restriction enzyme maps, pUK1, 3 and 4 showed overlapping with one another, they were considered to have cloned different regions of one and the same mRNA.

#### (10) Preparation of restriction enzyme maps

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The restriction enzyme maps were prepared for comparison for the four plasmids selected in the above.

The restriction enzymes used were

20 AccI, AluI, AvaI, Avall, BamHI, BclI, BglI, BglII, BstEII, ClaI, EcoRI, Fnu4HI, HaeIII, HincII, HindIII, KpnI, NcoI, MluI, PstI, PvuII, PsuI, SacI, Sall, Sau96I, Scal, SmaI, StuI, XbaI and XhoI. The buffers used with these restriction enzymes are shown in Table 5.

For AvaI, RsaI buffer was used; for SacI, KpnI buffer; for StuI, Sall and XhoI, BamHI buffer; for NcoI and Scal, BglII buffer; and, for MluI, HindIII buffer.

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Table 5

Enzyme	Final concentration of constituent						
	Tris mM	pH	NaCl mM	MgCl <sub>2</sub> mM	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> mM	KCl mM	Mercapto- ethanol
Acc I	10	7.5	60	7	—	—	5
Alu I	6	7.6	50	6			5
Ava II	6	8.0	60	10			5
Bam HI	10	8.0	100	7			5
Bcl I	6	7.4		10		75	—
Bgl I	10	7.4	66	10			2
Bgl II	10	7.5	100	10			5
Bst E II	6	7.9	150	6			—
Cla I	6	7.9	50	6			5
Eco RI	100	7.5	50	7	—	—	5
Fna 4 HI	6	7.4	6	6		5	5
Hae III	10	7.5	60	7			5
Hinc II	10	8.0	60	7			5
Hind III	10	7.5	60	7			5
Kpn I	6	7.5	6	6			—
Pst I	20	7.5	—	10	50	—	—
Pvu II	6	7.5	60	6			—
Rsa I	6	8.0	50	12			5
Sau 96 I	6	7.4	60	15			5
Sma I	6	8.0		6		20	5
Xba I	6	7.9	50	6			

As already mentioned hereinabove, the size of cDNA inserted in pUK1 is 1,900 bp and the largest among pUK1 through 4. The size of pUK3 and that of pUK4 are 1,300 bp and 1,150 bp, respectively. The cDNA of pUK2 is a fairly small and 170 bp in size. Therefore, for the three plasmids except pUK2, the restriction enzyme map of each cDNA was prepared. Table 6 show the number of restriction sites for each enzyme was determined (Table 6) and thus the distance between restriction enzyme sites was determined.

for each enzyme. Upon cleavage with EcoRI, pUK1 and pUK4 gave fragments quite equal in size to each other (420 bp). Upon double digestion with BamHI and EcoRI and with BamHI and PstI, a fragment from pUK1 was entirely the same in size with one from pUK3. Therefore, taking the EcoRI site as a basis for pUK1 and pUK4 and the BamHI site as a standard for pUK1 and pUK3, other restriction enzyme sites of these plasmids were examined, whereby the restriction enzyme sites on cDNA of pUK1 were found to be almost in agreement in position with those on cDNA of pUK3 and of pUK4. On the supposition that the cDNA of pUK1, that of pUK3 and that of pUK4 are identical to one another, it was estimated that, in pUK1 and pUK4, the cDNA was inserted in pBR322 in the same direction and that, in pUK3, the cDNA was inserted in the opposite direction as compared with pUK1 and pUK4.

Since the cDNAs inserted in pUK1, pUK3 and pUK4 could be regarded as one and the same in view of the restriction enzyme sites occurring therein, the possibility that these three cDNAs each might be the urokinase cDNA became very great. The grounds are that, in spite of very low probability of plasmids having a sequence identical with the base sequence of the synthetic 14-mer DNA used in screening, 3 out of the 4 colonies found to be positive in the screening had the same cDNA, rendering high the probability that the cDNA owned by these positive colonies might have the desired sequence for urokinase; and that the position of the DNA base sequence synthesized as a probe on urokinase mRNA (Fig. 3) agreed with the place of overlapping among pUK1, pUK3 and pUK4 (Fig. 1). The DNA base sequence of part of pUK1 and of pUK4 was determined. The corresponding amino acid sequence deduced was in agreement with the amino acid sequence of urokinase.

Table 6

Restriction enzyme	Number of restriction enzyme cleavage sites		
	p U K 1	p U K 3	p U K 4
A c c I	2	2	1
A v a I	0	0	0
B a m H I	1	1	0
B c l I	1	1	1
B g l I	0	0	1
B g l II	1	0	1
B s t E II	0	0	0
C l a I	0	0	0
E c o R I	2	1	2
H i n c II	0	0	0
H i n d III	0	0	0
N c o I	2	1	1
K p n I	0	0	0
M l u I	0	0	0
P s t I	2	3	1
P v u I	0	0	0
P v u II	4	3	2
R s a I	1	0	2
S a c I	0	0	0
S a l I	0	0	0
S c a I	0	0	1
S m a I	0	0	0
S t u I	1	1	0
X b a I	-	-	0
X h o I	0	-	0

(11) Determination of nucleotide sequence of urokinase cDNA

For the purpose of directly clarifying whether the plasmids obtained by the procedure mentioned in the preceding paragraph did contain the urokinase cDNA, the DNA base sequence was determined by the

Maxam-Gilbert method. Comparison of the possible amino acid sequence deducible from the DNA base sequence obtained with the known amino acid sequence of human urinary urokinase revealed that cloned cDNAs is a sequence for signal peptide-containing pro-UK.

(a) The materials and method used are as follows:

i) Plasmid DNA

The above-mentioned pUK1 and pUK4 were used.

ii) Restriction enzymes

AccI, BamHI, BglI, EcoRI, HpaI, PstI, PvuII, Sau3AI, SalI and RsaI were from Takara Shuzo and BclI, NcoI and TaqI from NEB.

iii) Restriction enzyme map of plasmid DNA

The restriction enzyme map of each plasmid with the cDNA inserted therein was prepared by the procedure mentioned under the preceding paragraph (10).

iv) Preparation of DNA fragment for use in base sequence determination

The DNA base sequence of each cDNA was determined by the Maxam-Gilbert sequencing method [Proc. Natl. Acad. Sci. U.S.A., 74, 560 (1977); Methods in Enzymol., 65, 499 (1980)].

Thus, the plasmid DNA was digested with an appropriate restriction enzyme and the 5'-end labelling was performed using [ $\gamma$ - $^{32}$ P]ATP and T4-polynucleotide Kinase (Fig. 5). The DNA fragments recovered were determined for its nucleotide sequence.

v) Sequencing of DNA nucleotide sequence

The [ $^{32}$ P]-labelled DNA fragment was subjected to Maxam-Gilbert's limited chemical degradation [Proc. Natl. Acad. Sci. U.S.A., 74, 560 (1977); Methods in Enzymol., 65, 499 (1980)], followed by 8% and 20% 7M urea-polyacrylamide gel electrophoresis in the conventional manner and autoradiography at -70°C for 2-3 days.

vi) Amino acid sequence of urokinase

Judgment as to whether the cDNA obtained was truly the urokinase cDNA was made by comparing the amino acid sequence deduced from the base sequence found with the known amino acid sequence of human urine urokinase [Hopper-Seyler's Z. physiol. Chem., 363, 1043 (1982)]. The following sequence (III) shows the total amino acid sequence each of human urokinase A-chain and B-chain.

Sequence (III): the entire amino acid sequence each of human urokinase chain A and chain B

A-Chain	
1	10
Ser-Asn-Glu-Leu-His-Glu-Val-Pro-Ser-Asn-Cys-Asp-Cys-Leu-Asn-Gly-Gly-Thr-Cys-Val-	20
21	30
Ser-Asn-Lys-Tyr-Phe-Ser-Asn-Ile-His-Trp-Cys-Asn-Cys-Pro-Lys-Lys-Phe-Gly-Gly-Gln-	40
41	50
His-Cys-Glu-Ile-Asp-Lys-Ser-Lys-Thr-Cys-Tyr-Glu-Gly-Asn-Gly-His-Phe-Tyr-Arg-Gly-	60
61	70
Lys-Ala-Ser-Thr-Asp-Thr-Met-Gly-Arg-Pro-Cys-Leu-Pro-Trp-Asn-Ser-Ala-Thr-Val-Leu-	80
81	90
Gln-Gln-Thr-Tyr-His-Ala-His-Arg-Ser-Asp-Ala-Leu-Gln-Leu-Gly-Lys-His-Asn-	100
101	110
Tyr-Cys-Arg-Asn-Pro-Asp-Asn-Arg-Arg-Pro-Trp-Cys-Tyr-Val-Gln-Val-Gly-Leu-Lys-	120
121	130
Pro-Leu-Val-Gln-Glu-Cys-Met-Val-His-Asp-Cys-Ala-Asp-Gly-Lys-Lys-Pro-Ser-Ser-Pro-	140
141	150
Pro-Glu-Glu-Leu-Lys-Phe-Gln-Cys-Gly-Gln-Lys-Thr-Leu-Arg-Pro-Arg-Phe- * * * *	160
B-Chain	
1	10
Ile-Ile-Gly-Gly-Glu-Phe-Thr-Thr-Ile-Glu-Asn-Gln-Pro-Trp-Phe-Ala-Ala-Ile-Tyr-Arg-	20
21	30
Arg-His-Arg-Gly-Gly-Ser-Val-Thr-Tyr-Val-Cys-Gly-Gly-Ser-Leu-Ile-Ser-Pro-Cys-Trp-	40

(continued on the following page)

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41 Val-Ile-Ser-Ala-Thr-His-Cys-Phe-Ile-Asp-Tyr-Pro-Lys-Lys-Glu-Asp-Tyr-Ile-Val-Tyr-  
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 61 Leu-Gly-Arg-Ser-Arg-Leu-Asn-Ser-Asn-Thr-Gln-Gly-Glu-Met-Lys-Phe-Glu-Val-Glu-Asn-  
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 81 Leu-Ile-Leu-His-Lys-Asp-Tyr-Ser-Ala-Asp-Thr-Leu-Ala-His-His-Asn-Asp-Ile-Ala-Leu-  
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 101 Leu-Lys-Ile-Arg-Ser-Lys-Glu-Gly-Arg-Cys-Ala-Gln-Pro-Ser-Arg-Thr-Ile-Gln-Thr-Ile-  
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 130  
 141 Gly-Lys-Glu-Asn-Ser-Thr-Asp-Tyr-Leu-Tyr-Pro-Glu-Gln-Leu-Lys-Met-Thr-Val-Val-Lys-  
 150  
 161 Leu-Ile-Ser-His-Arg-Glu-Cys-Gln-Gln-Pro-His-Tyr-Tyr-Gly-Ser-Glu-Val-Thr-Thr-Lys-  
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 181 Met-Leu-Cys-Ala-Ala-Asp-Pro-Gln-Trp-Lys-Thr-Asp-Ser-Cys-Gln-Gly-Asp-Ser-Gly-Gly-  
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 201 Pro-Leu-Val-Cys-Ser-Leu-Gln-Gly-Arg-Met-Thr-Leu-Thr-Gly-Ile-Val-Ser-Trp-Gly-Arg-  
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 221 Gly-Cys-Ala-Leu-Lys-Asp-Lys-Pro-Gly-Val-Tyr-Thr-Arg-Val-Ser-His-Phe-Leu-Pro-Trp-  
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 241 Ile-Arg-Ser-His-Thr-Lys-Glu-Glu-Asn-Gly-Leu-Ala-Leu  
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vii) Restriction enzyme map of cDNA and sequencing  
 strategy for determining DNA base sequence

pUK1 and pUK4 have restriction enzyme maps overlapping each other. On the other hand, pUK18,  
 which was selected by colony hybridization using the Nick-translated PstI fragment of pUK1, has a

restriction enzyme map overlapping with the map of pUK1 (Fig. 4). Therefore, the cDNAs of these plasmids are considered to have cloned different regions of the same mRNA and accordingly can be combinedly regarded as a single cDNA having a total length of about 2,250 base pairs, as shown in Fig. 5. According to this way of thinking, the sequencing strategy illustrated in Fig. 5 was established and the base sequence was determined.

[Results]

Comparison of the DNA base sequence obtained by sequencing with the amino acid sequence of human urinary urokinase [Sequence(III)] revealed that pUK1, 4 and 18 each contained part of pro-UK cDNA.

The following Sequence(IV) shows the total base sequence, from the 5'-end to the 3'-noncoding region, of the cDNA coding for an amino acid sequence equivalent to that of urokinase, as determined on the basis of the results of sequencing of individual fragments. Hereinafter, mention is made of the structure of the cDNA coding for an amino acid sequence equivalent to single-chain pro-urokinase as obtained in accordance with the present invention.

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-20 Met Arg Ala Leu Leu Ala Arg Leu Leu Cys Val Leu Val Val Ser Asp Ser Lys Gly  
 ATG AGA GCC CTG CTG GCG CGC CTG CTT CTC TGC GTC GTC GTC GTC GAC TCC AAA GGC  
 +1 10 20 30 40 50 60 70 80  
 AGCAATGAACCTTCATCAAGTTCATCGAACTGTGACGTCTAAATGGAGGAACATGTGTCTCCAAACAAGTACTTCTCCAA  
 Ser . . . . .  
 Mature UROKINASE  
 81 90 100 110 120 130 140 150 160  
 CATTCACTGGTGCAACTGCCCAAGAAATTCGGAGGCGCAGCACTGTGAAATAGATAAGTCAAAAACCTGCTATGAGGGGA  
 . . . . .  
 161 170 180 190 200 210 220 230 240  
 ATGGTCACCTTTTACCGAGGAAGGCCAGCACTGACACCATGGGCGGCCCTGCCCTGGAACCTGTGCCACTGTCTCTT  
 . . . . .  
 241 250 260 270 280 290 300 310 320  
 CAGCAAACGTACCATGCCCCACAGATCTGATGCTCTCTCAGCTGGGCCCTGGGAAACATAATTAATCTGCAGGAACCCAGACAA  
 . . . . .  
 321 330 340 350 360 370 380 390 400  
 CCGGAGGCGACCCCTGGTGCTATGTGCAGGTGGGCTTAAAGCCGCTTGTCCAAGAGTGCATGGTGCATGACTGCGCAGATG  
 . . . . .  
 401 410 420 430 440 450 460 470 480  
 GAAAAAGCCCTCCTCTCCAGAAATTAATAATTCAGTGTGGCCAAAAGACTCTGAGGCCCGCTTTAAGATTATT  
 . . . . .  
 481 490 500 510 520 530 540 550 560  
 GGGGGAGAATTACCAACCATCGAGAACCCAGCCCTGGTTTGGGCCCATCTACAGGAGGCCCGGGGGGCTCTGTACACCTA  
 . . . . .

(continued on the following page)

561 570 580 590 600 610 620 630 640  
 CGTGTGGAGGCCTCATCAGCCCTTGCTGGGTGATCAGGCCACACACTGCTTCATTGATTACCCAAAGAGGAGG  
 . . . . .  
 641 650 660 670 680 690 700 710 720  
 ACTACATCGTCTACCTGGGTCACTCAAGGCTTAACCTCCAACACGCAAGGGGAGATGAAGTTTGAGGTGGAACCTCATC  
 . . . . .  
 721 730 740 750 760 770 780 790 800  
 CTACACAAGGACTACAGCGCTGACACGCTTGCTCACCAACAACGACATTGCCCTTGCTGAAGATCCCGTTCCAAGGAGGGCAG  
 . . . . .  
 801 810 820 830 840 850 860 870 880  
 GTGTGGCAGCCATCCCGGACTATACAGACCATTGCTGCCCTGCCCTCGATGTATACGATCCCCAGTTGGCAAGCTGTG  
 . . . . .  
 881 890 900 910 920 930 940 950 960  
 AGATCACTGGCTTTGGAAAGAGAAATCTACCGACTATCTCTATCCGGAGCAGCTGAAGATGACTGTTGTGAAGCTGATT  
 . . . . .  
 961 970 980 990 1000 1010 1020 1030 1040  
 TCCCACCGGAGTGTACAGAGCCCCACTACTACGGCTCTGAAGTCAACCAACCAAAATGCTGTGTGCTGACCCACAGTG  
 . . . . .  
 1041 1050 1060 1070 1080 1090 1100 1110 1120  
 GAAACACAGATTCTGCCAGGGAGACTCAGGGGGACCCCTCGTCTGTTCCTCCAGGCCGATGACTTTGACTGGAATTG  
 . . . . .  
 1121 1130 1140 1150 1160 1170 1180 1190 1200  
 TGAGCTGGGCGGTGGATGTGCCCTGAAGGACAAGCCAGGCGTCTACAGAGAGTCTCACACTTCTTACCCCTGGATCCGC  
 . . . . .

(continued on the following page)

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molecule from the cell. The amino acid sequence beginning with the 21st amino acid, Ser, agreed with that of human urine urokinase A-chain.

In view of the above, it is evident that the cDNA obtained codes for pre-pro UK having a signal peptide comprising 20 amino acids.

ii) Cleavage site in pro-UK

Human urine urokinase, which is composed of two chains, A-chain and B-chain, is presumably formed by activation cleavage into two chains, under the action of protease, such as plasmin for instance, of a pro-urokinase molecule synthesized in and secreted from the cell. This was confirmed by sequencing of the cDNA of pUK1.

In sequence(II), it is seen that the carboxyl terminal amino acid sequence [the 157th amino acid the in Sequence (II)] of human urine urokinase A-chain is followed by Lys and further by the amino terminal amino acid sequence of B-chain, namely Ile-Ile-Gly-Gly-.....

This indicates that human urine urokinase is at first synthesized as a single-chain pro-urokinase composed of A-chain and B-chain coupled with other via Lys and that said pro-urokinase is cleaved at the Arg-Phe-Lys-Ile-Ile site with loss of Lys.

iii) Carboxyl terminal and 3'-noncoding region of cDNA

Sequence(II) shows the terminal portion of the coding region of the cDNA provided by the present invention and part of the 3'-noncoding region thereof. As shown in the Sequence(II), the amino acid sequence agreeing with the carboxyl terminal of human urine urokinase B-chain, such as Gly-Leu-Ala-Leu, is read out from this base sequence and, downstream therefrom, there occurs the protein synthesis termination codon TGA. Therefore, it was considered that the carboxyl terminal of human urine urokinase is identical with that of pro-UK according to the invention and that there is no polypeptide to be lost by processing.

On the other hand, the restriction enzyme map suggests that the 3'-noncoding region should be huge. Although only part of the 3'-noncoding region has so far been sequenced, the 3'-noncoding region hitherto obtained in the study of pUK18 is about 850 bp in size. However, pUK18 does not contain the poly(A) sequence characteristic of the 3'-terminal of eukaryotic mRNA, so that the 3'-noncoding region is possibly much longer.

(12) Construction of plasmid containing urokinase-coding region

pUK1 and pUK4 were digested with the restriction enzymes HindIII and BglII at 37°C for 1-2 hours according to the table in paragraph (10). A fragment of about 5.3 kb was isolated from pUK1, and a fragment of about 1.2 kb from pUK4. Both the fragments were ligated with each other by reaction in ligation buffer (20 mM Tris-HCl pH 7.5, 10 mM MgCl<sub>2</sub>, 10 mM dithiothreitol, 0.5 mM ATP) containing 10 units of T4 DNA ligase at 20°C for 2 hours. The above steps are shown in Fig. 6. (In Fig. 6, E stands for EcoRI, H for HindIII, and B for BamHI). Resulted plasmid, named pUK33, containing about 2.2 kbp of pre-urokinase cDNA.

(13) Production of urokinase in animal cells

(a) Construction of vector for expression in animal cells

pSV-GI (Fig. 8) was constructed using, as the starting plasmids, pcDVI and pLI (Fig. 7) created by Okayama and Berg [Molec. and Cell. Biol., 3, 280-289 (1983)]. The pcDVI and pLI are hybrid plasmids of pBR322 DNA and SV40 DNA. They were obtained from PL Biochemicals (Pharmacia). For facilitating the insertion of urokinase-encoding DNA into these plasmids, pSV-GI was constructed by inserting a HindIII-PstI fragment of pLI into pcDVI at the HindIII-KpnI site thereof.

Thus, 4 µg of pLI was digested with PstI, the protruding 3'-end was rendered blunt by treatment with T4 DNA polymerase. Namely, 76 µl of reaction mixture No. 1 (4 µg of pLI, 8 µl of 10 x T4 polymerase buffer, 4.0 µl each of 2 mM dNTP solution, d H<sub>2</sub>O) was heated at 65°C for 3 minutes and then quenched, 4 µl (10 U) of T4 polymerase was added to make the total volume 80 µl, and the reaction was allowed to proceed at 37°C for 5 minutes. Thereafter, 8 µl of 0.25 M EDTA (pH 8.0) and 80 µl of d H<sub>2</sub>O were added, the resulting mixture was subjected once to treatment with phenol-chloroform, and the aqueous layer was recovered. DNA was recovered by precipitation with ethanol, dried under reduced pressure, and subjected to KpnI linker ligation. The ligation reaction was carried out at 16°C overnight in a reaction mixture No. 2 (4 µg of dried DNA, 2 µl (2 µg) of 5'-P KpnI linker, 2.0 µl of 10 x ligase buffer, d H<sub>2</sub>O) adding 2 µl (5 U) of T4 ligase. Thereafter, digestion was carried out with HindIII, followed by 5% polyacrylamide gel electrophoresis (PAGE), whereby a DNA fragment of about 600 bp

was recovered.

Separately, pcDVI was digested with HindIII and KpnI, and a DNA fragment of about 2.7 kbp was isolated on 1% agarose gel. A 100-ng portion of the fragment was ligated with the above-mentioned pLI-derived DNA fragment of about 600 bp. The ligation was carried out by adding 2  $\mu$ l (5.6 U) of T4 ligase to 8  $\mu$ l of reaction mixture No. 3 (150 ng of pLI fragment DNA, 100 ng of pcDVI fragment DNA, 1.0  $\mu$ l of 10 x T4 ligase buffer, d H<sub>2</sub>O) and allowing the reaction to proceed at 16 °C overnight. A 1/2 portion of the reaction mixture was used for performing transformation of *E. coli* HB101.

Using 12 colonies out of the transformants obtained, plasmid DNAs were prepared by the mini-prep method and examined by digestion with various restriction enzymes. All the plasmid DNAs were found to contain the desired plasmid. Therefore, using one strain, plasmid DNAs were prepared in large amounts and named pSV-GI, the restriction enzyme map thereof was prepared (Fig. 8). The pSV-GI obtained could be judged to have SV-40 early enhancer/promoter element and late 5'-splicing junction arranged upstream and late poly(A) signal and terminator arranged downstream, with the KpnI site as the cloning site and thus have elements involved in the functional mRNA synthesis in animal cells without any deficiency.

(b) Insertion of urokinase DNA sequence into vector

pUK33 (containing urokinase cDNA 2.2 kbp in total length) obtained in (12) was partially digested with PstI, and a 1.7 kbp fragment was isolated and inserted into KpnI site of the pSV-GI. Thus, 500  $\mu$ l of reaction mixture No. 4 [100  $\mu$ g of pUK33, 50  $\mu$ l of 10 x PstI buffer, 10  $\mu$ l (60 U) of PstI, d H<sub>2</sub>O] was maintained at 37 °C. After 10, 15 and 20 minutes of reaction, the reaction mixture was sampled in about 160  $\mu$ l portions. The sampled reaction mixtures were heat-treated at 65 °C for 5 minutes, combined and subjected to 1% agarose gel electrophoresis, whereby about 10  $\mu$ g of the 1.7 kbp containing pre-UK cDNA with its 5'-and 3'-noncoding sequence fragment was recovered. About 5  $\mu$ g of the recovered fragment of about 1.7 kbp was treated with T4 DNA polymerase to render the protruding 3'-end blunt, and subjected to ligation with KpnI linker as in (13-a). The ligation product was digested with KpnI and then further subjected to ligation with KpnI-digested pSV-GI. The product was used for transformation of *E. coli* HB101, whereby pSV-GI-preUK (Fig. 9) was obtained.

As seen in Fig. 10 pSV-GI-preUK contains 5'-noncoding region of pro-UK cDNA, signal sequence and 3'-noncoding region as inserted downstream from SV-40 early promoter, and when introduced into appropriate recipient cells, the DNA can induce production of human pro-UK.

(c) Preparation of dominant selective marker-containing plasmid

Using the above pSV-GI, pSV-GI-Neo<sup>r</sup>, a plasmid to be used as a dominant selective marker in transforming cells to be cultured, was constructed in the following manner.

The plasmid pNEO with the Tn5-derived Neo<sup>r</sup> gene clones therein [Southern, P. J. and Berg, J.: Molec. and Applied Genet., 1, 327-341 (1982)] was subjected to double digestion with BamHI and HindIII, followed by recovery of DNA fragments by precipitation with ethanol. The DNAs recovered were rendered blunt-ended by treatment with *E. coli* DNA polymerase I large fragment (Klenow fragment) in the presence of all 4 dNTPs. A blunt-ended 1.5 kb DNA fragment was separated and then the KpnI linker was ligated using T4 DNA ligase. After KpnI linker ligation, the DNA fragment was completely digested with 60 U of KpnI, and the desired Neo<sup>r</sup> fragment of about 1.5 kb with said linker joined thereto was recovered.

A 150-ng portion of the DNA fragment thus recovered was ligated with 20 ng of pSV-GI cleaved with KpnI in the above manner, and the ligation product was used for transformation of *E. coli* HB101. In this manner, pSV-GI-Neo<sup>r</sup> was obtained (Fig. 9).

(d) Preparation of recipient cell

Chinese hamster ovary cell [CHO-K1, ATCC CCL61 (purchased from Flow Labo. Inc.)] was used as the recipient cells. Cell culture was conducted in Ham's F-12 (Gibco) containing 10% FCS (fetal calf serum) (Boehringer Mannheim). The cells were maintained by subculture in Falcon 3028 flasks. Subconfluent cells were collected 2 days after inoculation by trypsinization, suspended in the above-mentioned culture medium and inoculated into Falcon #3003 (100 mm) culture dishes at an inoculum size of about  $5 \times 10^5$  cells/10ml/100mm. After 24 hours of incubation in a CO<sub>2</sub> incubator (5% CO<sub>2</sub>-95% air), the cells were subjected to transformation. The number of cells per dish on that occasion was about  $7.0 \times 10^5$ .

(e) Transformation

To the dish prepared in (d), there was added 1 ml per dish of a DNA CaPO<sub>4</sub> CO- precipitate containing 2.0  $\mu$ g (as DNA)/ml of the plasmid DNA [pSV-GI-preUK and pSV-GI-Neo (mixing ratio 100:1)] and 20  $\mu$ g/ml of sonicated salmon sperm DNA as a carrier DNA, as prepared by the method of Osada et al. [Protein, Nucleic Acid and Enzyme, 28 (14), 1569-1581 (1983)]. About 18 hours after exposure to

DNA, the medium was exchanged. The incubation was then continued further 48 hours or more, followed by selection using antibiotics G-418.

(f) Selection of transformant cells

Since the urokinase gene itself is not "selectable", the transformant selection in eukaryotic cells was performed by cultivating the cells in G-418-containing medium (400  $\mu$ g/ml) according to the method of Southern and Berg. [J. Molec. and Applied Genet., 1, 327-341 (1982)], with the Tn5-derived Neo<sup>r</sup> gene capable of giving G-418 resistance as the dominant selection marker. The G-418 used was from Gibco (Geneticin, Gibco, lot No. 75K6043 and 74N8040).

While exchanging the medium every 4 days, G-418-resistant colonies were cloned and grown. On day 10, 83 such colonies were obtained. The colonies obtained were examined for production of urokinase with RPHA reagent for human urokinase (Green Cross Corp.) and by fibrin plate assay. Primary screening with RPHA reagent revealed that about 25% of the G-418-resistant colonies produced human urokinase-like substance.

Fifteen strains showing RPHA-aggregation were tested for plasminogen activation activity by fibrin plate assay, whereby the activity was observed in the culture supernatant. This activity was neutralized by antihuman urokinase antibody purified by urokinase antigen column chromatography. The strain CHO-KI-G-68 seemingly highly capable of producing urokinase-like substance as estimated in the screening was chosen and re-cloned by low density culture, and two strains C-68-53 and C-68-61 were obtained.

Using the culture supernatant of these strains, the human urokinase-like substance synthesized and secreted was examined for its properties. The clones C-68-53 and C-68-61 secreted human urokinase-like protein into the medium in a stable and constitutive manner for more than 100 days.

(g) Properties of human urokinase-like protein synthesized by CHO-KI cells

For said two clones, the human urokinase-like protein secreted into the medium was examined for its properties.

First, the production of human urokinase-like protein by each of these two clones was determined. Thus, each clone was inoculated into 25 cm<sup>2</sup> culture flasks (Falcon #3013) at an inoculum size of  $5 \times 10^5$  cells/10 ml/flask. When the cells became confluent after 3 days of incubation, the medium was replaced with 5 ml/flask of maintenance medium containing 1% of FCS. After 24 hours of incubation in maintenance medium, the plasminogen activator activity in the culture supernatant was measured. The activity measurement by the fibrin plate method using human urinary high molecular weight urokinase as a reference indicated a yield of about 200 IU/ml/day for both the clones. Such activity was specifically neutralized by antihuman urokinase monoclonal antibody.

Next, the molecular weight of human urokinase-like protein produced by said clones was determined. Thus, the culture supernatant after 24 hours of incubation in maintenance medium was subjected to 12.5% SDS-PAGE. The band was blotted onto a nitrocellulose filter electrophoretically in 25 mM Tris-192 mM glycine (pH 8.3)/20% methanol. The filter was then blocked in 20 mM Tris-HCl (pH 7.5)/500mM NaCl containing 3% of gelatin at room temperature for 60 minutes and subjected to treatment with rabbit antihuman urokinase IgG purified by means of a human urokinase antigen column and protein A Sepharose as a primary antibody. Thus the human Urokinase band was specifically identified by using immuno-Blot<sup>TM</sup> assay system (Bio-Rad).

When the samples were used after reduction treatment with 2-mercaptoethanol, the culture supernatants of both the clones gave three bands, a main band 54 K in molecular weight and subbands 34 K and 20 K in molecular weight. Simultaneous electrophoresis of human urine-derived two-chain high molecular UK (as a reference) and the culture supernatant of pro-UK-producing human kidney-derived cell line revealed that the 54 K, 34 K and 20 K bands from the culture supernatant from both C-68-53 and C-68-61 cells agreed in molecular weight and reactivity to anti-UK IgG with the human natural-type single-chain pro-UK (54 K) band and the 34 K and 20 K bands from double-chain high molecular UK, respectively, no distinction could be made. (Fig. 11)

The above results indicated that, in both the clones, glycosylated single-chain pro-UK having a molecular weight of 54 K is synthesized and secreted, like in the case of said natural type, and part of it is converted secondarily to double-chain UK in the culture medium.

Therefore, the presence or absence of the sugar chain in the human pro-UK synthesized and secreted by both the clones was then examined by column chromatography using ConA-Sepharose 4B (Pharmacia).

Thus, the clones were cultivated until cells became confluent. The cells were washed twice with Dulbecco's PBS (phosphate-buffered Saline) (+) (Nissui) and then grown in 5 ml of proline-added, Met-free Eagle's MEM medium (product of Nissui) containing 1% of FCS dialyzed against PBS(+), together with 200  $\mu$ Ci/ml of [<sup>35</sup>S]-methionine, for 20 hours.

The [<sup>35</sup>S]-labelled culture fluid was applied to an anti-human urokinase monoclonal antibody column (5 ml). The column was washed well with 0.04 M sodium phosphate buffer (pH 8.0)/ 0.03 M NaCl and then eluted with glycine-HCl (pH 7.5), whereby <sup>35</sup>S-labelled recombinant human urokinase was recovered. The eluate <sup>35</sup>S-labelled human urokinase was neutralized with 1 M Tris, dialyzed against 20 mM sodium phosphate buffer pH 7.4/150 mM NaCl and applied to a ConA-Sepharose 4B (Pharmacia) column. After washing with buffer, elution was performed with sodium phosphate buffer (pH 7.4) containing 0.1 M methyl- $\alpha$ -D-mannoside. Most of the radioactivity eluted from the anti-UK monoclonal antibody column was adsorbed on ConA-Sepharose and specifically eluted with mannoside.

The above results indicate that the recombinant urokinase synthesized and secreted by both the clones is a glycoprotein purifiable by means of an anti-human urokinase monoclonal antibody column.

The above results also indicate that the human pro-UK cDNA introduced into CHO-K1 can be transcribed in a normal manner and the pro-UK is synthesized and secreted as a glycoprotein having a molecular weight equivalent to that of the natural-type pro-UK and having reactivity to monoclonal anti human Urokinase antibodies.

#### Claims

1. A method of producing glycosylated single-chain pro-urokinase which comprises cultivating animal cells transformed with a plasmid with the DNA inserted therein, said DNA having the sequence

Sequence(I): the base sequence of the structural gene for pro-UK

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1      10      20
Ser-Asn-Glu-Leu-His-Gln-Val-Pro-Ser-Asn-Cys-Asp-Cys-Leu-Asn-Gly-Gly-Thr-Cys-Val-
AGC AAT GAA CTT CAT CAA GTT CCA TCG AAC TGT GAC TGT CTA AAT GGA GGA ACA TGT GTG

21      30      40
Ser-Asn-Lys-Tyr-Phe-Ser-Asn-Ile-His-Trp-Cys-Asn-Cys-Pro-Lys-Lys-Phe-Gly-Gly-Gln-
TCC AAC AAG TAC TTC TCC AAC AAT CAC TGG TGC AAC TGC CCA AAG AAA TTC GGA GGG CAG

41      50      60
His-Cys-Glu-Ile-Asp-Lys-Ser-Lys-Thr-Cys-Tyr-Glu-Gly-Asn-Gly-His-Phe-Tyr-Arg-Gly-
CAC TGT GAA ATA GAT AAG TCA AAA ACC TGC TAT GAG GGG AAT GGT CAC TTT TAC CGA GGA

61      70      80
Lys-Ala-Ser-Thr-Asp-Thr-Met-Gly-Arg-Pro-Cys-Leu-Pro-Trp-Asn-Ser-Ala-Thr-Val-Leu-
AAG GCC AGC ACT GAC ACC ATG GGC CGG CCC TGC CTG CCC TGG AAC TCT GCC ACT GTC CTT

81      90      100
Gln-Gln-Thr-Tyr-His-Ala-His-Arg-Ser-Asp-Ala-Leu-Gln-Leu-Gly-Leu-Gly-Lys-His-Asn-
CAG CAA ACG TAC CAT GCC CAC AGA TCT GAT GCT CTT CAG CTG GGC AAA CAT AAT

101     110     120
Tyr-Cys-Arg-Asn-Pro-Asp-Asn-Arg-Arg-Arg-Pro-Trp-Cys-Tyr-Val-Gln-Val-Gly-Leu-Lys-
TAC TGC AGG AAC CCA GAC AAC CGG AGG CGA CCC TGG TGC TAT GTG CAG GTG GGC CTA AAG

121     130     140
Pro-Leu-Val-Gln-Glu-Cys-Met-Val-His-Asp-Cys-Ala-Asp-Gly-Lys-Lys-Pro-Ser-Ser-Pro-
CCG CTT GTC CAA GAG TGC ATG GTG CAT GAC TGC GCA GAT GGA AAA AAG CCC TCC TCT CCT

141     150     160
Pro-Glu-Glu-Leu-Lys-Phe-Gln-Cys-Gly-Gln-Lys-Thr-Leu-Arg-Pro-Arg-Phe-Lys-Ile-Ile-
CCA GAA GAA TTA AAA TTT CAG TGT GGC CAA AAG ACT CTG AGG CCC CGC TTT AAG ATT ATT

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(continued on the following page)



161 170 180  
 Gly-Gly-Glu-Phe-Thr-Ile-Glu-Asn-Gln-Pro-Trp-Phe-Ala-Ala-Ile-Tyr-Arg-Arg-His-  
 GGG GGA GAA TTC ACC ACC ATC GAG AAC CAG CCC TGG TTT GCG GCC ATC TAC AGG AGG CAC  
 181 190 200  
 Arg-Gly-Gly-Ser-Val-Thr-Tyr-Val-Cys-Gly-Ser-Leu-Ile-Ser-Pro-Cys-Trp-Val-Ile-  
 CGG GGG GGC TCT GTC ACC TAC GTG TGT GGA GGC AGC CTC ATC AGC CCT TGC TGG GTG ATC  
 201 210 220  
 Ser-Ala-Thr-His-Cys-Phe-Ile-Asp-Tyr-Pro-Lys-Lys-Glu-Asp-Tyr-Ile-Val-Tyr-Leu-Gly-  
 AGC GCC ACA CAC TGC TTC ATT GAT TAC CCA AAG AAG GAG GAC TAC ATC GTC TAC CTG GGT  
 221 230 240  
 Arg-Ser-Arg-Leu-Asn-Ser-Asn-Thr-Gln-Gly-Glu-Met-Lys-Phe-Glu-Val-Glu-Asn-Leu-Ile-  
 CGC TCA AGG CTT AAC TCC AAC ACG CAA GGG GAG ATG AAG TTT GAG GTG GAA AAC CTC ATC  
 241 250 260  
 Leu-His-Lys-Asp-Tyr-Ser-Ala-Asp-Thr-Gln-Ala-His-Asn-Asp-Ile-Ala-Leu-Leu-Lys-  
 CTA CAC AAG GAC TAC AGC GCT GAC ACG CTT GCT GCT CAC CAC AAC GAC ATT GCC TTG CTG AAG  
 261 270 280  
 Ile-Arg-Ser-Lys-Glu-Gly-Arg-Cys-Ala-Gln-Pro-Ser-Arg-Thr-Ile-Gln-Thr-Ile-Cys-Leu-  
 ATC CGT TCC AAG GAG GGC AGG TGT GCG CAG CCA TCC CGG ACT ATA CAG ACC ATC TGC CTG  
 281 290 300  
 Pro-Ser-Met-Tyr-Asn-Asp-Pro-Gln-Phe-Gly-Thr-Ser-Cys-Glu-Ile-Thr-Gly-Phe-Gly-Lys-  
 CCC TCG ATG TAT AAC GAT CCC CAG TTT GGC ACA AGC TGT GAG ATC ACT GGC TTT GGA AAA  
 301 310 320  
 Glu-Asn-Ser-Thr-Asp-Tyr-Leu-Tyr-Pro-Glu-Gln-Leu-Lys-Met-Thr-Val-Val-Lys-Leu-Ile-  
 GAG AAT TCT ACC GAC TAT CTC TAT CCG GAG CAG CTG AAG ATG ACT ACT GTT CTG AAG CTG ATT  
 321 330 340  
 Ser-His-Arg-Glu-Cys-Gln-Gln-Pro-His-Tyr-Tyr-Gly-Ser-Glu-Val-Thr-Thr-Lys-Met-Leu-  
 TCC CAC CGG GAG TGT CAG CAG CCC CAC TAC TAC GGC TCT GAA GTC ACC ACC AAA ATG CTG

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341 Cys-Ala-Ala-Asp-Pro-Gln-Trp-Lys-Thr-Asp-Ser-Cys-Gln-Gly-Asp-Ser-Gly-Gly-Pro-Leu- 360  
 TGT GCT GCT GAC CCA CAG CAG TGG AAA ACA GAT TCC TGC CAG GGA GAC TCA GGG GGA CCC CTC  
 350  
 361 Val-Cys-Ser-Leu-Gln-Gly-Arg-Met-Thr-Leu-Thr-Gly-Ile-Val-Ser-Trp-Gly-Arg-Gly-Cys- 380  
 GTC TGT TCC CTC CAA GGC CGC ATG ACT TTG ACT GGA ATT GTG AGC TGG GGC CGT GGA TGT  
 370  
 381 Ala-Leu-Lys-Asp-Lys-Pro-Gly-Val-Tyr-Thr-Arg-Val-Ser-His-Phe-Leu-Pro-Trp-Ile-Arg- 400  
 GCC CTG AAG GAC AAG CCA GGC GTC TAC ACG AGA GTC TCA CAC TTC TTA CCC TGG ATC CGC  
 390  
 401 Ser-His-Thr-Lys-Glu-Glu-Asn-Gly-Leu-Ala-Leu 410  
 AGT CAC ACC AAG GAA GAG AAT GGC CTG GCC CTC

and being prepared by using as the template mRNA obtained from cells of an established human kidney-derived cell line.

2. A method of producing glycosylated single-chain pro-urokinase as claimed in Claim 1, wherein the glycosylated single-chain pro-urokinase has a molecular weight of about 54,000.
3. A method of producing glycosylated single-chain pro-urokinase according to Claims 1 and 2, wherein

the animal cells are Chinese hamster ovary cells.

# Revendications

1. Procédé de préparation de pro-uro-kinase glycosylée simple-brin, qui comprend la culture de cellules animales transformées en présence d'un plasmide contenant le DNA correspondant, ledit DNA ayant la séquence :

Sequence (I) : la séquence de base du gène de structure pour pro-UK

```

1      20
Ser-Asn-Glu-Leu-His-Gln-Val-Pro-Ser-Asn-Cys-Asp-Cys-Leu-Asn-Gly-Gly-Thr-Cys-Val-
AGC AAT GAA CTT CAT CAA GTT CCA TCG AAC TGT GAC TGT CTA AAT GGA GGA ACA TGT GTG

21      40
Ser-Asn-Lys-Tyr-Phe-Ser-Asn-Ile-His-Trp-Cys-Asn-Cys-Pro-Lys-Lys-Phe-Gly-Gly-Gln-
TCC AAC AAG TAC TTC TCC TCC NAC ATT CAC TGG TGC AAC TGC CCA AAG AAA TTC GGA GGG CAG

31      60
His-Cys-Glu-Ile-Asp-Lys-Ser-Lys-Thr-Cys-Tyr-Glu-Gly-Asn-Gly-His-Phe-Tyr-Arg-Gly-
CAC TGT GAA ATA GAT AAG TCA AAA ACC TGC TAT GNG GGG AAT GGT CAC TTT TAC CGA GGA

41      80
Lys-Ala-Ser-Thr-Asp-Thr-Met-Gly-Arg-Pro-Cys-Leu-Pro-Trp-Asn-Ser-Ala-Thr-Val-Leu-
AAG GCC AGC ACT GAC ACC ATG GGC CGG CCC TGC CTG CCC TGG AAC TCT GCC ACT GTC CTT

51      100
Gln-Gln-Thr-Tyr-His-Ala-His-Arg-Ser-Asp-Ala-Leu-Gln-Leu-Gly-Lys-His-Asn-
CAG CAA ACG TAC CAT GCC CAC AGA TCT GAT GCT CTT CAG CTG GGC CTG GGG AAA CAT AAT

61      120
Tyr-Cys-Arg-Asn-Pro-Asp-Asn-Arg-Arg-Pro-Trp-Cys-Tyr-Val-Gln-Val-Gly-Leu-Lys-
TAC TGC AGG AAC CCA GAC AAC CGG AGG CGA CCC TGG TGC TAT GTG CAG CTG GGC CTA AAG

71      140
Pro-Leu-Val-Gln-Glu-Cys-Met-Val-His-Asp-Cys-Ala-Asp-Gly-Lys-Lys-Pro-Ser-Ser-Pro-
CCG CTT GTC CAA GAG TGC ATG GTG CAT GAC TGC GCA GAT GGA AAA AAG CCC TCC TCT CCT

81      160
Pro-Glu-Glu-Leu-Lys-Phe-Gln-Cys-Gly-Gln-Lys-Thr-Leu-Arg-Pro-Arg-Phe-Lys-Ile-Ile-
CCA GAA GAA TTA AAA TTT CAG TGT GGC CAA AAG ACT CTG AGG CCC CGC TTT AAG ATT ATT

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(suite sur la page suivante)

161 Gly-Gly-Glu-Phe-Thr-Thr-Ile-Glu-Asn-Gln-Pro-Trp-Phe-Ala-Ile-Tyr-Arg-Arg-His-180  
 GGG GGA GAA TTC ACC ACC ATC GAG AAC CAG CCC TGG TTT GCG GCC ATC TAC AGG AGG CAC  
 181 Arg-Gly-Gly-Ser-Val-Thr-Tyr-Val-Cys-Gly-Gly-Ser-Leu-Ile-Ser-Pro-Cys-Trp-Val-Ile-200  
 CCG GGG GGC TCT GTC ACC TAC GTG TGT GGA GGC AGC CTC ATC AGC CCT TGC TGG GTG ATC  
 201 Ser-Ala-Thr-His-Cys-Phe-Ile-Asp-Tyr-Pro-Lys-Lys-Glu-Asp-Tyr-Ile-Val-Tyr-Leu-Gly-220  
 AGC GCC ACA CAC TGC TTC ATT GAT TAC CCA AAG AAG CAG GAC TAC ATC GTC TAC CTG GGT  
 221 Arg-Ser-Arg-Leu-Asn-Ser-Asn-Thr-Gln-Gly-Glu-Met-Lys-Phe-Glu-Val-Glu-Asn-Leu-Ile-240  
 CGC TCA AGG CTT AAC TCC AAC ACG CAA GGG GAG ATG AAG TTT GAG GTG GAA AAC CTC ATC  
 241 Leu-His-Lys-Asp-Tyr-Ser-Ala-Asp-Thr-Leu-Ala-His-His-Asn-Asp-Ile-Ala-Leu-Leu-Lys-260  
 CTA CAC AAG GAC TAC AGC GCT GAC ACG CTT GCT CAC CAC AAC GAC ATT GCC TTG CTG AAG  
 261 Ile-Arg-Ser-Lys-Glu-Gly-Arg-Cys-Ala-Gln-Pro-Ser-Arg-Thr-Ile-Gln-Thr-Ile-Cys-Leu-280  
 ATC CGT TCC AAG GAG GGC AGG TGT GCG CAG CCA TCC CGG ACT ATA CAG ACC ATC TGC CTG  
 281 Pro-Ser-Met-Tyr-Asn-Asp-Pro-Gln-Phe-Gly-Thr-Ser-Cys-Glu-Ile-Thr-Gly-Phe-Gly-Lys-300  
 CCC TCG ATG TAT AAC GAT CCC CAG TTT GCG ACA AGC TGT GAG ATC ACT GGC TTT GGA AAA  
 301 Glu-Asn-Ser-Thr-Asp-Tyr-Leu-Tyr-Pro-Glu-Gln-Leu-Lys-Met-Thr-Val-Val-Lys-Leu-Ile-320  
 GAG AAT TCT ACC GAC TAT CTC TAT CCG GAG CAG CTG AAG ATG ACT CTT GTG AAG CTG ATT  
 321 Ser-His-Arg-Glu-Cys-Gln-Gln-Pro-His-Tyr-Tyr-Gly-Ser-Glu-Val-Thr-Thr-Lys-Met-Leu-340  
 TCC CAC CGG GAG TGT CAG CAG CCC CAC TAC TAC GGC TCT GAA GTC ACC ACC AAA ATG CTG

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341 Cys-Ala-Ala-Asp-Pro-Gln-Tip-Lys-Thr-Asp-Ser-Cys-Gln-Gly-Asp-Ser-Gly-Gly-Pro-Leu-  
 TGT GCT GCT GAC CCA CAG TGG AAA ACA GAT TCC TGC CAG GGA GAC TCA GGG GGA CCC CTC 360

350  
 361 Val-Cys-Ser-Leu-Gln-Gly-Arg-Met-Thr-Leu-Thr-Gly-Ile-Val-Ser-Trp-Gly-Arg-Gly-Cys-  
 GTC TGT TCC CTC CAA GGC CGC ATG ACT TTG ACT GGA ATT GTG AGC TGC GGC CGT GGA TGT 380

370  
 381 Ala-Leu-Lys-Asp-Lys-Pro-Gly-Val-Tyr-Thr-Arg-Val-Ser-His-Phe-Leu-Pro-Trp-Ile-Arg-  
 CCC CTG AAG GAC AAG CCA GGC GTC TAC ACG AGA GTC TCA CAC TTC TTA CCC TGG ATC CGC 400

390  
 401 Ser-His-Thr-Lys-Glu-Glu-Asn-Gly-Leu-Ala-Leu  
 AGT CAC ACC AAG AAG GAA GAG AAT GGC CTG GCC CTC 410

et étant préparé par l'utilisation d'un brin mRNA matrice, obtenu à partir de cellules d'une lignée cellulaire établie dérivant du rein humain.

2. Procédé de préparation de pro-urokinase glycosylée simple brin selon la revendication 1, caractérisé en ce que la pro-uro-kinase glycosylée simple brin a un poids moléculaire d'environ 54 000.
3. Procédé de préparation de pro-uro-kinase glycosylée simple brin selon l'une des revendications 1 et 2, caractérisé en ce que les cellules animales sont des cellules d'ovaires d'hamsters chinois.

## Patentansprüche

1. Verfahren zur Herstellung glycosylierter einkettiger Pro-Urokinase, umfassend die Kultivierung von Tierzellen, die mit einem Plasmid mit der eingesetzten DNA transformiert wurden, wobei die DNA die Sequenz

Sequenz (I): Basensequenz des Strukturgens für Pro-UK

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1      20
Ser-Asn-Glu-Leu-His-Gln-Val-Pro-Ser-Asn-Cys-Asp-Cys-Leu-Asn-Gly-Gly-Thr-Cys-Val-
AGC AAT GAA CTT CAT CAA GTT CCA TCG AAC TGT GAC TGT CTA NAT GGA GGA ACA TGT GTG

21      40
Ser-Asn-Lys-Tyr-Phe-Ser-Asn-Ile-His-Trp-Cys-Asn-Cys-Pro-Lys-Lys-Phe-Gly-Gly-Gln-
TCC AAC AAG TAC TAC TTC TCC AAC ATT CAC TGG TGC AAC TGC CCA AAG AAA TTC GGA GGG CAG

41      60
His-Cys-Glu-Ile-Asp-Lys-Ser-Lys-Thr-Cys-Tyr-Glu-Gly-Asn-Gly-His-Phe-Tyr-Arg-Gly-
CAC TGT GAA ATA GAT AAG TCA AAA ACC TGC TAT GAG GGG AAT GGT CAC TTT TAC CGA GGA

61      80
Lys-Ala-Ser-Thr-Asp-Thr-Met-Gly-Arg-Pro-Cys-Leu-Pro-Trp-Asn-Ser-Ala-Thr-Val-Leu-
AAG GCC AGC ACT GAC ACC ATG GGC CGG CCC TGC CTG CCC TGG AAC TCT GCC ACT GTC CTT

81      100
Gln-Gln-Thr-Tyr-His-Ala-His-Arg-Ser-Asp-Ala-Leu-Gln-Leu-Gly-Leu-Gly-Lys-His-Asn-
CAG CAA ACG TAC CAT GCC CAC CAC NGA TCT GAT GCT CTT CAG CTG GGC CTG GGG AAA CAT AAT

101     120
Tyr-Cys-Arg-Asn-Pro-Asp-Asn-Arg-Arg-Arg-Pro-Trp-Cys-Tyr-Val-Gln-Val-Gly-Leu-Lys-
TAC TGC AGG AAC CCA GAC AAC CGG AGG CGA CCC TGG TGC TAT GTG CAG GTG GGC CTA AAG

121     140
Pro-Leu-Val-Gln-Glu-Cys-Met-Val-His-Asp-Cys-Ala-Asp-Gly-Lys-Lys-Pro-Ser-Ser-Pro-
CCG CTT GTC CAA GAG TGC ATG GTG CAT GAC TGC GCA GAT GGA AAA AAG CCC TCC TCT CCT

141     160
Pro-Glu-Glu-Leu-Lys-Phe-Gln-Cys-Gly-Gln-Lys-Thr-Leu-Arg-Pro-Arg-Phe-Lys-Ile-Ile-
CCA GAA GAA TTA AAA TTT CAG TGT GGC CAA AAG ACT CTG AGG CCC CGC TTT AAG ATT ATT

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(Fortsetzung nächste Seite)

161 Gly-Gly-Glu-Phe-Thr-Thr-Ile-Glu-Asn-Gln-Pro-Trp-Phe-Ala-Ile-Tyr-Arg-Arg-His- 170 180  
 GGG GGA GAA TTC ACC ACC ATC GAG AAC CAG CCC TGG TTT GCG GCC ATC TAC AGG AGG CAC  
 181 Arg-Gly-Gly-Ser-Val-Thr-Tyr-Val-Cys-Gly-Gly-Ser-Leu-Ile-Ser-Pro-Cys-Trp-Val-Ile- 190 200  
 CCG GGG GGC TCT GTC ACC TAC GTG TGT GGA GGC AGC CTC ATC AGC CCT TGC TGG GTG ATC  
 201 Ser-Ala-Thr-His-Cys-Phe-Ile-Asp-Tyr-Pro-Lys-Lys-Glu-Asp-Tyr-Ile-Val-Tyr-Leu-Gly- 210 220  
 AGC GCC ACA CAC TGC TTC ATT GAT TAC CCA AAG AAG GAG GAC TAC ATC GTC TAC CTG GGT  
 221 Arg-Ser-Arg-Leu-Asn-Ser-Asn-Thr-Gln-Gly-Glu-Met-Lys-Phe-Glu-Val-Glu-Asn-Leu-Ile- 230 240  
 CGC TCA AGG CTT AAC TCC MAC ACG CAA GGG GAG ATG AAG TTT GAG GTG GAA AAC CTC ATC  
 241 Leu-His-Lys-Asp-Tyr-Ser-Ala-Asp-Thr-Leu-Ala-His-Asn-Asp-Ile-Ala-Leu-Leu-Lys- 250 260  
 CTA CAC AAG GAC TAC AGC GCT GAC ACG CTT GCT CAC CAC AAC GAC ATT GCC TTG CTG AAG  
 261 Ile-Arg-Ser-Lys-Glu-Gly-Arg-Cys-Ala-Gln-Pro-Ser-Arg-Thr-Ile-Gln-Thr-Ile-Cys-Leu- 270 280  
 ATC CGT TCC AAG GAG GGC AGG TGT GCG CAG CCA TCC CGG ACT ATA CAG ACC ATC TGC CTG  
 281 Pro-Ser-Met-Tyr-Asn-Asp-Pro-Gln-Phe-Gly-Thr-Ser-Cys-Glu-Ile-Thr-Gly-Phe-Gly-Lys- 290 300  
 CCC TCG ATG TAT AAC GAT CCC CAG TTT GGC ACA AGC TGT GAG ATC ACT GGC TTT GGA AAA  
 301 Glu-Asn-Ser-Thr-Asp-Tyr-Leu-Tyr-Pro-Glu-Gln-Leu-Lys-Met-Thr-Val-Val-Lys-Leu-Ile- 310 320  
 GAG AAT TCT ACC GAC TAT CTC TAT CCG GAG CAG CTG AAG ATG ACT GGT GTG AAG CTG ATT  
 321 Ser-His-Arg-Glu-Cys-Gln-Gln-Pro-His-Tyr-Tyr-Gly-Ser-Glu-Val-Thr-Thr-Lys-Met-Leu- 330 340  
 TCC CAC CGG GAG TGT CAG CAG CCC CAC TAC TAC GGC TCT GAA CTC ACC ACC AAA ATG CTG

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341 Cys-Ala-Ala-Asp-Pro-Gln-Trp-Lys-Thr-Asp-Ser-Cys-Gln-Gly-Asp-Ser-Gly-Gly-Pro-Leu-  
 TGT GCT GCT GAC CCA CAG TGG AAA ACA GAT TCC TGC CAG GGA GAC TCA GGG GGA CCC CTC 360

361 Val-Cys-Ser-Leu-Gln-Gly-Arg-Met-Thr-Leu-Thr-Gly-Ile-Val-Ser-Trp-Gly-Arg-Gly-Cys-  
 GTC TGT TCC CTC CAA GGC CGC ATG ACT TTG ACT GGA ATT GTG AGC TGG GGC CGT GGA TGT 380

381 Ala-Leu-Lys-Asp-Lys-Pro-Gly-Val-Tyr-Thr-Arg-Val-Ser-His-Phe-Leu-Pro-Trp-Ile-Arg-  
 GCC CTG AAG GAC AAG CCA CCA GGC GTC TAC ACG AGA GTC TCA CAC TTC TTA CCC TGG ATC CGC 400

401 Ser-His-Thr-Lys-Glu-Glu-Asn-Gly-Leu-Ala-Leu  
 AGT CAC ACC AAG GAA GAG AAT GGC CTG GCC CTC 410

besitzt und hergestellt wird durch Verwendung einer mRNA als Matrize, die aus Zellen einer etablierten, aus Human-Nieren abgeleiteten Zelllinie erhalten wurden.

- 55 2. Verfahren zur Herstellung glycosylierter einkettiger Pro-Urokinase nach Anspruch 1, wobei die glycosylierte einkettige Pro-Urokinase ein Molekulargewicht von etwa 54 000 besitzt.
3. Verfahren zur Herstellung glycosylierter einkettiger Pro-Urokinase nach den Ansprüchen 1 oder 2,



wobei es sich bei den Tierzellen um Ovar-Zellen von chin sischen Hamstern handelt.

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FIG. 1

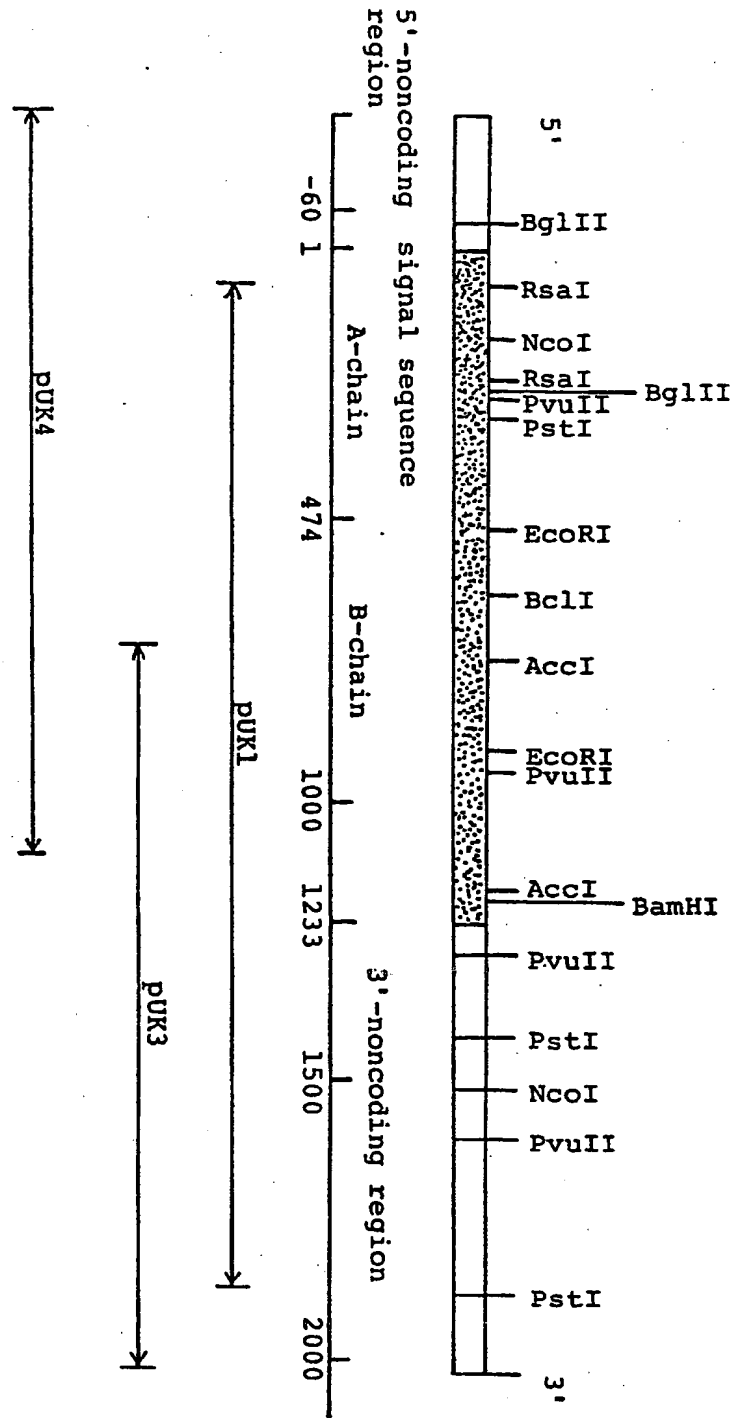


FIG. 2

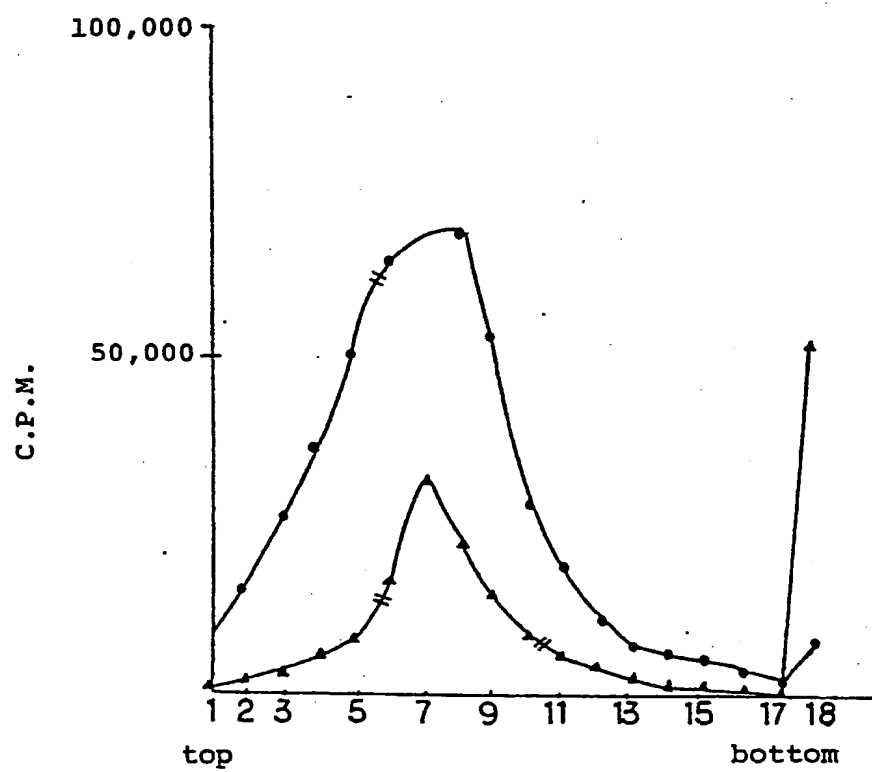


FIG.3

Glu-Met-Lys-Phe-Glu

codon 5'-GA<sup>G</sup><sub>A</sub>-AUG-AA<sup>G</sup><sub>A</sub>-UU<sup>U</sup><sub>C</sub>GA-3'

(probe 3'-CT<sup>T</sup><sub>C</sub>TACTT<sup>T</sup><sub>C</sub>AA<sup>G</sup><sub>A</sub>CT-5' )

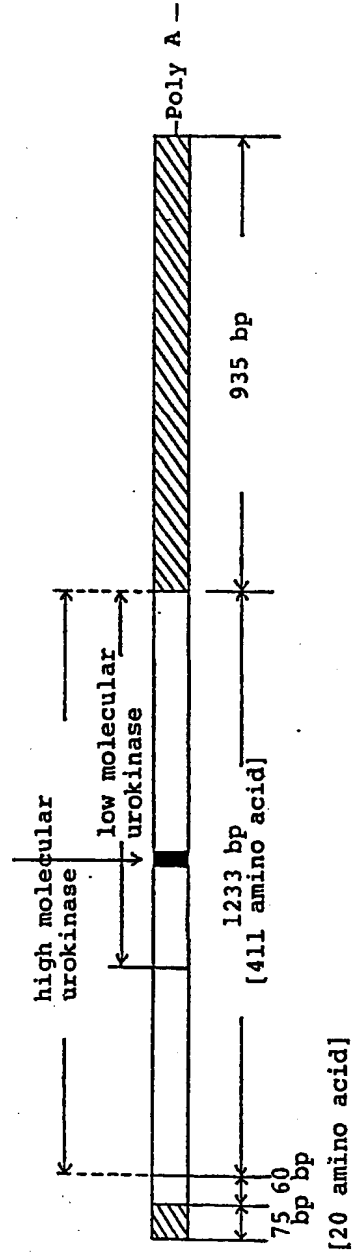


FIG.4

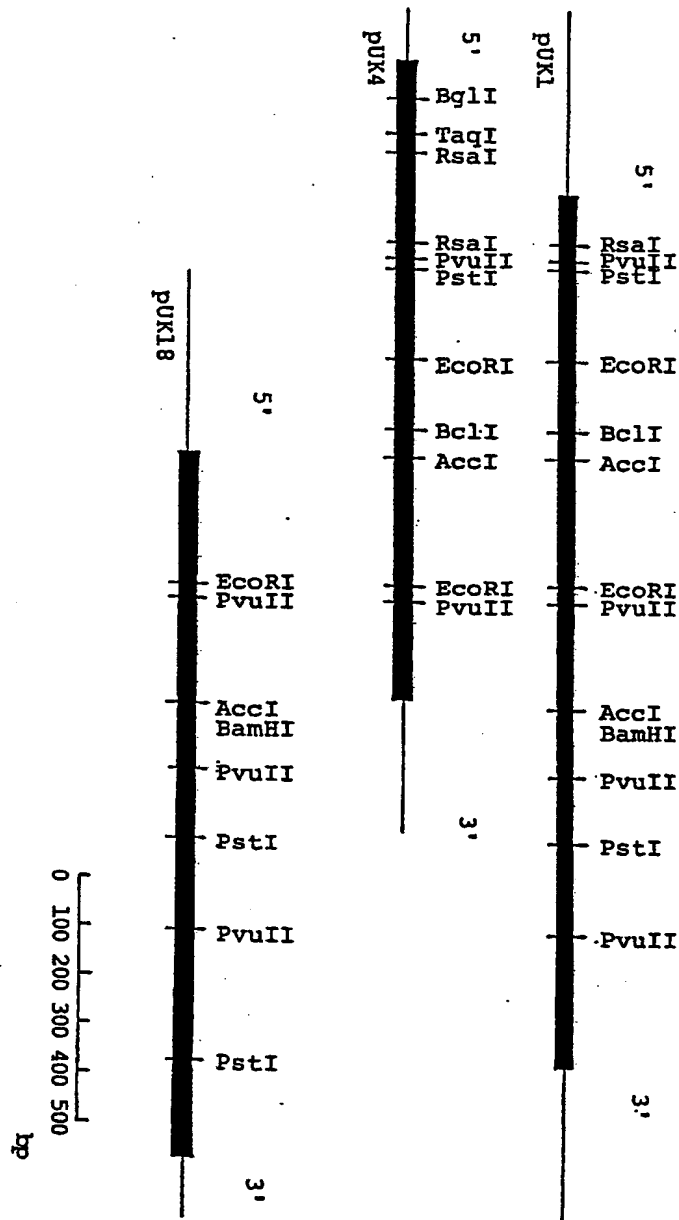


FIG. 5

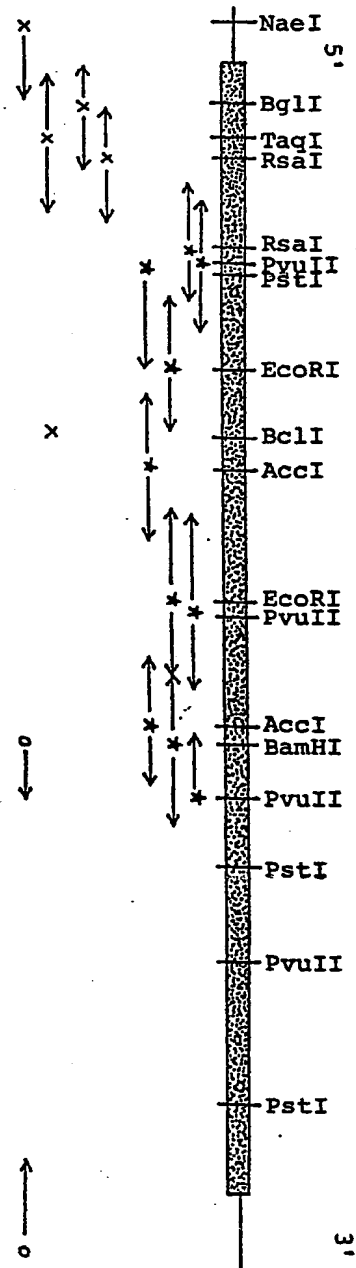


FIG. 6

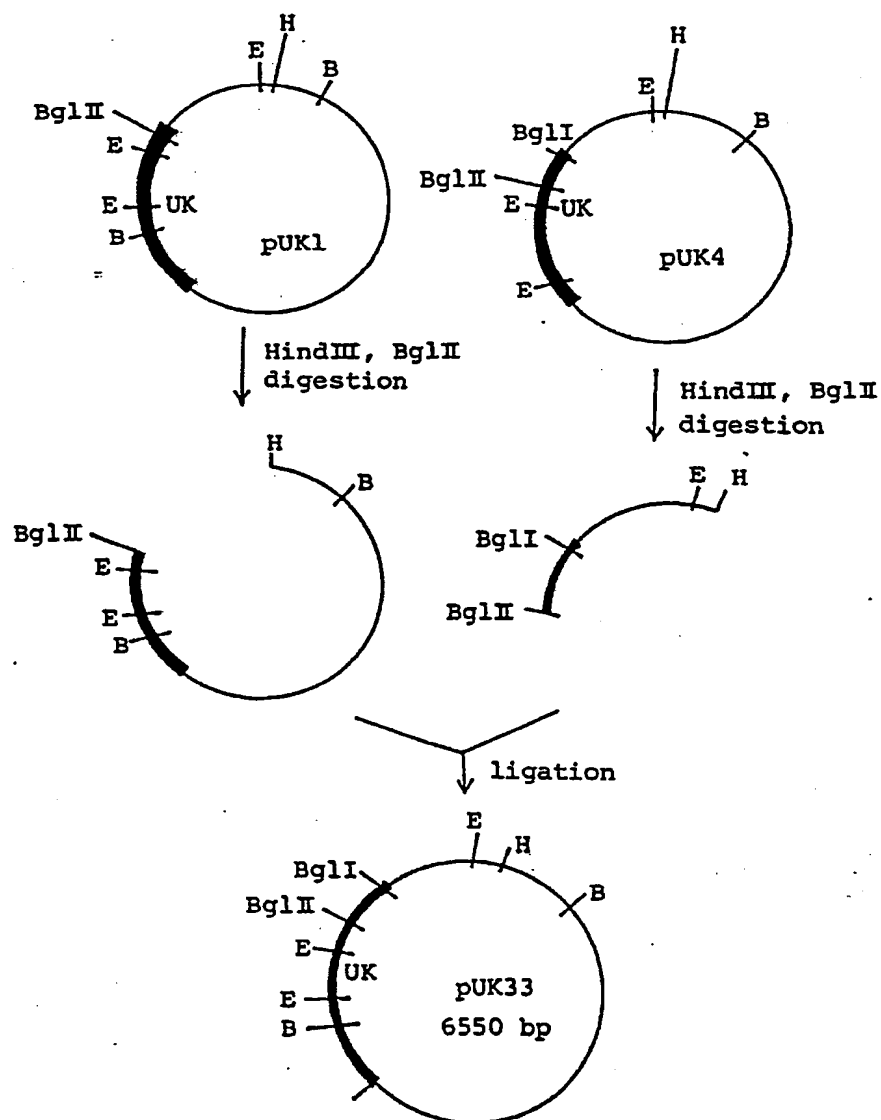


FIG. 7

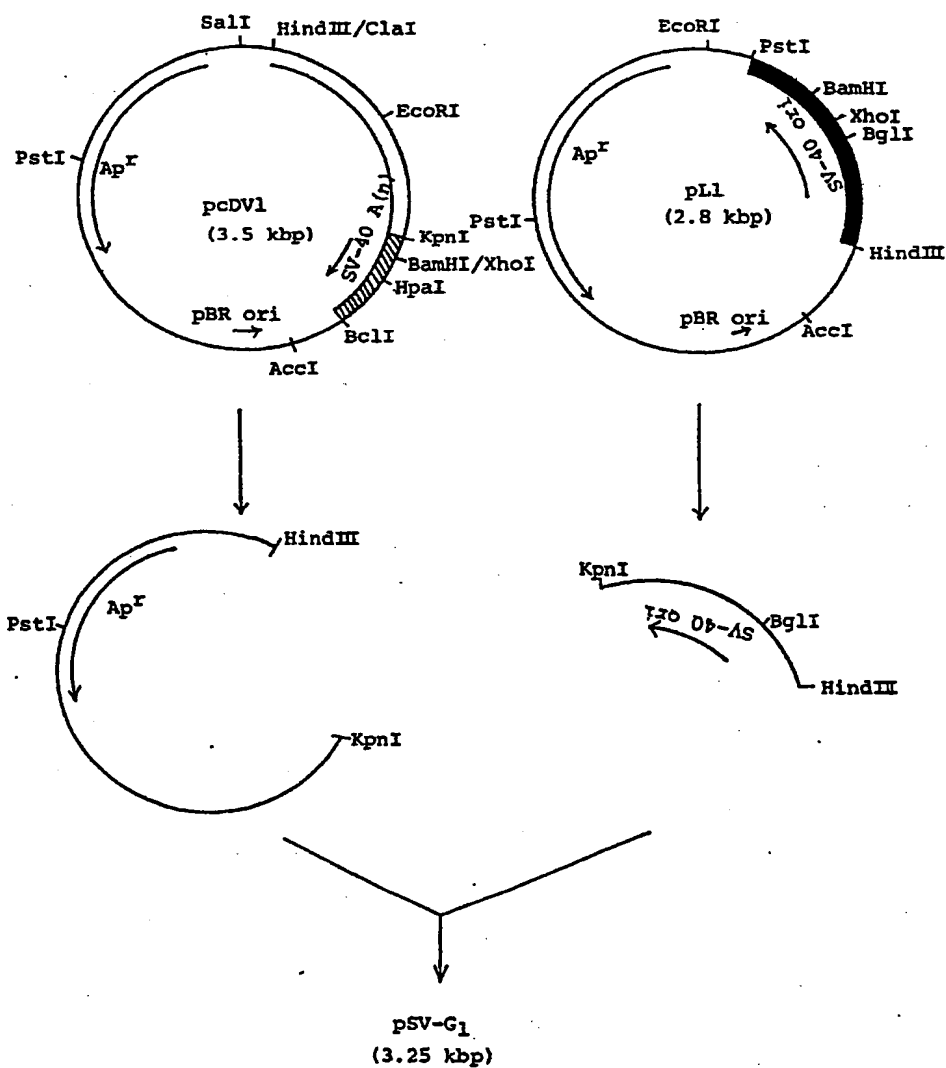




FIG. 8

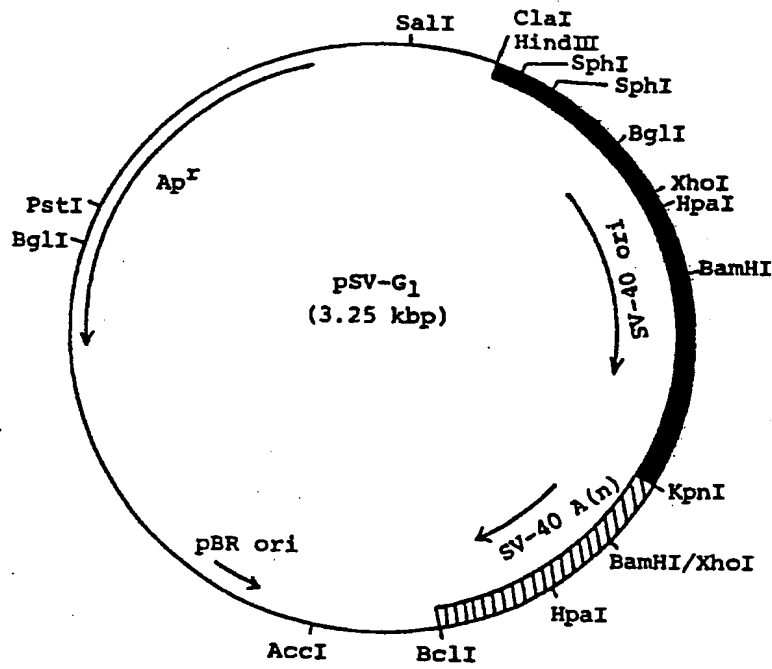


FIG. 10

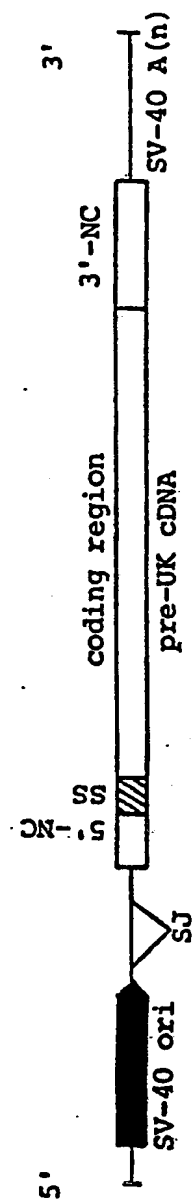


FIG. 11

